ANTIMICROBIAL SUSCEPTIBILITY TESTING THROUGH IMAGE ANALYSIS

Dissertation submitted to

The Tamil Nadu Dr. M. G. R. Medical University, Chennai

in partial fulfillment of the award of degree of

MASTER OF PHARMACY

(PHARMACEUTICAL BIOTECHNOLOGY)

Submitted by

MUNAVIR.M.K.

Under the guidance of

Dr.D.C. SUNDARAVELAN, M. Pharm., Ph.D.

Department of Pharmaceutical Biotechnology

MARCH – 2009

COLLEGE OF PHARMACY

SRI RAMAKRISHNA INSTITUTE OF PARAMEDICAL SCIENCES

COIMBATORE – 641 044.

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This is to certify that the dissertation entitled **"ANTIMICROBIAL SUSCEPTIBILITY TESTING THROUGH IMAGE ANALYSIS"** was carried out by **Mr. MUNAVIR.M.K.,** in the Department of Pharmaceutical Biotechnology, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, which is affiliated to **The Tamil Nadu Dr.M.G.R. Medical University, Chennai**, under supervision and direct guidance of **Dr.D.C.Sundaravelan, M.Pharm, Ph.D.** Department of Pharmaceutical Biotechnology, College of Pharmacy, SRIPMS, Coimbatore – 44.

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Munavir MK

CONTENTS

INTRODUCTION

To manage and treat patients with infectious disease intelligently, the physician must know specifically which organism is the causative and which drug at which concentration is curative. In diagnosing infectious diseases, then it is necessary to isolate and identify the specific causative organism, of infectious and to predict which drug is active against it. In general clinical laboratory procedures have come about as a result of a very thorough knowledge of the physiological behavior of the different microorganism and the curative agent against them. Tests that identify disease producing microorganism and suitable drugs demonstrate the value to the medical world of a good knowledge of 'microbial physiology'. The portion of microbiology that deals with procedures for diagnosing infectious diseases is generally known as clinical microbiology.

Many times the physicians, when treating an infection, wishes to know not only which organism is involved but also what antibiotics may be used to manage the infection. Many antibiotics lose their effectiveness against certain kinds of bacteria and therefore it becomes very necessary to test each specific infection, using organisms taken from that infection, against a series of potentially effective antibiotics. Even if the identity of the organism is known antibiotic susceptibility test must be performed.

The well or disc diffusion method of antibiotic susceptibility evaluation is deceptively simple on the surface and tends to mask the variables that must be considered in making a clear evaluation.

The ability of the antibiotic to diffuse is in a state of dynamic interaction with the rapidly growing population of bacteria. The antimicrobial is diffusing in a ring of ever-diminishing concentration through the bacterial growth, and a point is reached where a given concentration is just sufficient to stop further bacterial growth. Therefore these types of assay only should only be employed with bacteria considered to be a rapidly growing species, i.e. those capable of growing into a visible mat of growth on an agar

surface in 12 to 18 hours.

A standardized technique, known as the Kirby Bauer method, is now widely used in clinical microbiology laboratories to evaluate the effectiveness of antibiotics on organisms isolated from infections. This technique has the advantage of predicting the clinical effectiveness of a given drug with reasonable accuracy by noting the diameter of the zones of inhibition and comparing this data with a standard chart (L. Jack Bradshaw, 1979).

But as technology developed the early detections of the zones of inhibition through image analysis is possible. This can be done by several ways.

DETERMINATION OF ANTIBIOTIC ACTIVITY

After a pathogen is cultured its sensitivity to specific antibiotics serves as a guide in choosing antimicrobial therapy. Some pathogen such as ***Streptococcus pyrogen*** and ***Neisseria meningitides***, usually have predictable sensitivity patterns to certain antibiotics. Most gram negative bacilli, enterococci, and staphylococcal species often show unpredictable sensitivity pattern to various antibiotics, and require susceptibility testing to determine appropriate antimicrobial therapy. The disk diffusion method is useful when susceptibility to an unusual antibiotic, not available in automated systems is to be determined (William A Strohl et al., 2001).

Classical method of assaying antibiotics is the agar zone diffusion technique in which there is a linear relationship between the response and the dose. This technique is still widely used, even though it suffers from the disadvantages that it is unsatisfactory when results are needed rapidly (A Denver Russell et al., 1983).

METHODS

Kirby-Bauer Antimicrobial Sensitivity Test

The drug sensitivities of many pathogenic microorganisms are known, but it is sometimes necessary to test several agents to determine the drug of choice. A standardized filter-paper disc-agar diffusion procedure, known as the Kirby-Bauer method, is frequently used to determine the drug sensitivity of microorganisms isolated from infectious processes. This method allows for the determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surround the disc. In this procedure, filter-paper discs of uniform size are impregnated with specified concentrations of different antibiotics and then placed on the surface of an agar plate that has been seeded with the organism to be tested. The medium of choice is Mueller-Hinton agar, with a pH of 7.2 to 7.4, which is poured into plates to a uniform depth of 5 mm and refrigerated on solidification. The plates are then heavily inoculated with a standardized inoculom by means of a cotton swab to ensure the confluent growth of the organism.

The plates are examined for the presence of growth inhibition, which is indicated by a clear zone surrounding each dis. The susceptibility of an organism to a drug is determined by the size of this zone, which itself is dependent on variables such as:

1. The ability and rate of diffusion of the antibiotic into the medium and its interaction with the test organism.
2. The number of organisms inoculated.
3. The growth rate of the organism.
4. The degree of sensitivity of the organism to the antibiotic.

A measurement of the diameter of the zone of inhibition in millimeters is made and its size is compared to the contained in a standardized chart. Based on this comparison, the test organism is determined to be resistant, intermediate or sensitive to the antibiotic (James G Cappuccino, 1990).

Serial Dilution Tube Technique

Serial dilution tests in which the concentration gradient is discontinuous are frequently used to determine the antibiotic sensitivity of strains bacteria and to assay the antibiotic activity of body fluids during treatment.

To each of a series of sterile stopper test tube a standard volume of medium that will support the growth and the test organism is added. Solution of the antimicrobial agent is prepared in broth and a serious doubling dilution prepared with sterile pipettes. The range of concentrations should extend from at level twice the highest concentration likely to be found in the tissues during treatment to half that which inhibits the growth of the most sensitive member of the species being tested compare with tubes contain (a) medium without antibiotic and (b) uninoculated medium. The inoculum, consisting of a suitable dilution of an overnight broth culture of the test or standard control organism, is added, one loopful to each tube.

The tubes are incubated at 37 degree C, for 24 hour and examined for turbidity. The tube with the highest dilution showing no visible turbidity is minimum inhibitory concentration (bacteriostatic concentration). To measure the bactericidal concentration it is necessary to subculture from the tubes showing no visible growth on the agar or into broth free of the microbial agent. The highest dilution is bring no growth is the bactericidal concentration. Where available, a special neutralizer of the antimicrobial agent should be used in the subculture medium (Robert Cruickshan K, 1972).

THEORY OF ANTIBIOTIC INHIBITION ZONE FORMATION

Zone size is the sequence of two dynamic systems proceeding simultaneously. First is the diffusion of the antibiotic from a confined source and the second, growth of the test organism. Using an established formula for the diffusion of neutral particles in gases, Cooper & Woodman (1946) demonstrated its application to the diffusion

antibiotics through agar gels. The formula may be written:

$$X^2 = 4\ DT\ 2.3\ (\log m_o - \log m^1)$$

in which X = the distance between the source of the antibiotic and the edge of the zone of inhibition (i.e. the zone size); D = the diffusion coefficient of the antibiotic; T = the time taken for the zone to be fixed; m_o = the antibiotic concentration at source; m^1 = the critical concentration of the antibiotic which inhibits the test organism under conditions of diffusion.

Diffusion Coefficient

An antibiotic diffuses into an agar gel from a point source at a constant rate depending on the chemical and physical properties of the gel and the ionic charge on diffusing molecules. Two factors significantly affect this constant.

(1) Temperature. Forces which bring about diffusion of molecules are proportional to the absolute temperature. Most microbiological assays are carried out under uniform temperature conditions and the diffusion coefficient for a given antibiotic is likely to be relatively constant).

(2) Viscosity of the solvent. The charge on the solvent molecules set up a resistance to the diffusing molecules and therefore reduces the rate of diffusion. Most assays use water as solvent but other solvent may strongly influence the diffusion coefficient. If other substances are dissolved in the antibiotic solution (e.g. sugars) this too will have an effect on the diffusion coefficient.

The concentration of antibiotic diffusing from a reservoir and the size of the zone of inhibition (X)

The antibiotic concentration and the size of the inhibition zone are both dependent on each other. Under standard conditions the size of the zone is directly related to the concentration of the antibiotic being used. High concentrations at source drive an inhibitory concentration (m^1) of the antibiotic further out into the agar in time T. This

results in large zones being produced. Low concentrations do not drive the inhibitory concentration so far out into the agar and the zones are correspondingly smaller. Where the difference between m_o and m' is large the concentration gradient is steep and the zone edge tends to be crisp: where they are similar the resultant shallow concentration gradient may be in diffuse zone edges.

Under the normal conditions of assay, m_o may be considered to be constant throughout the period T. However, in disc susceptibility tests, the concentration of antibiotic at source is not known. The disc is impregnated with a known amount of the drug but this dissolves in an unknown volume of water in the sensitivity agar. Hence the concentration cannot be determined and, for this reason, it is not possible to relate directly the sizes of zones produced by solutions of known concentrations with discs of known amounts (A Denver Russell et al., 1983).

Critical inhibitory concentration of the antibiotic (m^1 and MIC)

For each antibiotic there is a minimum concentration (m^1) diffusing from the reservoir which inhibits each test organism under conditions of diffusion. This is constant for each organism: it can be determined by extrapolation and, being independent of many factors such as temperature of incubation and inoculum size, is one of the most accurate determinations of sensitivity.

Invariably the m^1 value is 2 to 4 times greater than the minimum inhibitory concentration (MIC) for the same organism. The methods by which each is determined, however, are intrinsically different. MICs are determined by exposing the organism directly to the antibiotic at different concentrations from time zero: the m' value is the inhibitory concentration of the antibiotic reaching the growing organism with time by diffusion.

The time (T) required for the zone edge to be formed

Usually zones are measured after at least overnight incubation. The zone however, is fixed very much earlier, usually after a few hours from time 0. Thereafter the test organism grows up outside the zone to render of the edge of the zone visible and measurable. The critical time (T) is the required for the critical inhibitory concentration (m^1) to reach the position in the agar where the zone edge will be formed. This coincides certain density of bacteria.

Growth of the test organism

Time T is also the time required for the inoculum to reach a certain bacterial density by growth. From the moment of incubation the inoculum on seeded agar plate follows the normal laws of growth. After an initial lag phase, the inoculum moves fairly rapidly into a log phase of growth having a relatively constant doubling time. The time taken to reach the critical density will be dependent on many factors, including the biological nature of the organism under test, and particularly its doubling time, the nutritional status of the medium, the temperature of incubation and the size of the inoculum used. With dense inocula the time required to reach this critical density will be much shorter than with a light inoculum. Also, if very dense inocula are used, no zones of inhibition will be obtained even with high concentration of antibiotics. This illustrates the fundamental difference between diffusion and incorporation methods of sensitivity testing (A Denver Russell et al., 1983).

Growth of Microorganism

Growth in a bacterial cell involves coordinated increase in the mass of its constituent parts; it is not simply an increase in total mass since this could be due, for example, to the accumulation of a storage compound within the cell.

Usually, growth leads to the division of a cell into two similar or identical cells.

Thus, growth and reproduction are closely linked in bacteria, and the term ‘growth’ is generally used to cover both processes.

Factors affecting growth of microorganism

Bacteria grow only if their environment is suitable; if it’s not optimal, growth may occur at a lower rate or not all – or the bacteria may die, depending on species and conditions.

Essential requirements for growth include (i) s supply of suitable nutrients; (ii) a source of energy; (iii) water (iv) an appropriate temperature; (v) an appropriate pH; (vi) appropriate levels (or the absence) of oxygen(Paul Singleton et al., 1995).

Methods of identifying organisms for diagnosis purpose

- ❖ Direct microscopic visualizations
- ❖ Cultivation and identification
- ❖ Detection of microbial antigens
- ❖ Detection of microbial RNA or DNA
- ❖ Detection of host immune response

ACTIONS ON PETRI PLATE

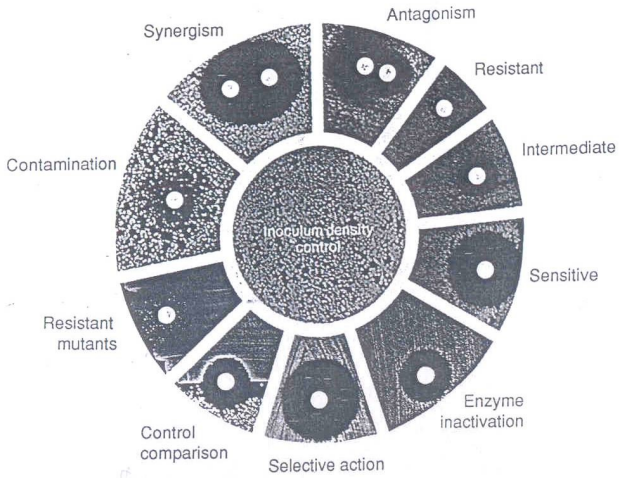


FIG :1

Resistant

No zone: growth occurs right up to the disc.

Intermediate

A narrow growth-free zone surrounds the disc.

Sensitive

A wide growth-free zone surrounds the disc.

Enzyme inactivation

A narrow growth free zone surrounds the disc. Unlike the intermediate zone, the edge of the zone is sharply defined and it contains somewhat heavier growth with some normal-sized or relatively large colonies.

Selective Action

This result can be obtained e.g. when the inoculum consists of two different strains which differ in their degree of susceptibility to the given antibiotic. Close to the disc, the concentration of antibiotic is high enough to inhibit both strains (narrow growth-free zone). Further from the disc (lower concentration of antibiotic) one strain is still inhibited while the other can grow.

Control Comparison

The ‘half-zone’ obtained with a control strain (one side of the disc) is opposite the ‘half-zone’ obtained with the test strain (other side of the disc). To make this comparison, the test and control strains are inoculated onto separate halves of the plate and the disc is placed between them.

Resistant Mutants

The inoculum contained a small proportion of mutant cells which were able to form colonies under conditions which inhibited non-mutants. The mutants are usually antibiotic-resistant cells. There is, however, another type of mutant which grows only in the presence of a given antibiotic, and such mutants would also form colonies in an otherwise growth-free zone; for example, 'streptomycin dependent' mutants contain non-functional ribosomes which, in the presence of streptomycin appear to distorted in such a way that they become functional.

Contamination

The inoculum contained a mixture of organisms, at least one of which is resistant to the antibiotic.

Synergism

The two discs contain different antibiotics. The zone shows an inhibitory effect which is greater than the sum of the effects of each antibiotic acting alone; that is, the antibiotics are acting synergically.

Antagonism

The two discs contain different antibiotics. Here, the presence of one antibiotic inhibits the activity of the other (Paul Singleton et al., 1995).

FACTORS INFLUENCING DIFFUSION TESTS

Many variables influence the final result of antimicrobial diffusion tests in different ways at the same time. The more important variables that are readily subjected to experimental control are outlined below:

i. Inoculum density
ii. Visualization of the zone edge
iii. Agar depth

iv. Composition of the agar medium
v. Growth characteristics of the test strain
vi. Temperature of Incubation
vii. Incubation time
viii. Timing of drug application
ix. Concentration of antimicrobial agents in the reservoir
x. Presence of serum proteins

(i) Inoculum density

The size of inoculums is the single most important variable that influences the results of susceptibility tests. The position of the zone of inhibition is determined when the critical cell mass is obtained. More time is required to reach that cell mass when the inoculums is light; consequently, the critical concentration of drug can diffuse further, resulting is larger zones of inhibition. Heavy inocula tent to give small zones of inhibition. For susceptibility testing moderately heavy inoculums of about 10^6 viable cells per 15cm Petri Plate is generally recommended.

(ii) Visualization of the zone edge

Zones of inhibition on susceptibility test plates can not be measured with extreme degrees of precision. In all situations it is very important to standardize the intensity and angle of light used to illuminate the test plates when zone measurements are being determined.

(iii) Agar depth

When agar layers are no more than 2 to 3 mm in depth, very slight variations will have profound effects on the zone sizes. For susceptibility testing, an agar depth of about 4mm is recommended.

(iv) Composition of the agar medium

The agar medium itself profoundly influences the zone sizes in three ways

1. It affects the activity of different antimicrobial agents
2. It influences the rate of diffusion of the antimicrobial agent
3. It affects the growth rate of the test organism.

(v) Growth characteristics of the test strain

The rate of growth on the test medium obviously affects the end result. In susceptibility testing, some strain-to strain variability in growth rate sis unavoidable. Growth conditions have been standardized for optimal results with most of the common rapid-growing bacterial pathogens.

(vi) Temperature of Incubation

To further complicate the situation, most antimicrobial agents diffuse more slowly at lower temperatures, partially because of the increased viscosity of the agar medium. A single plate placed on the metal shelf of an incubator may take about 1 hour to warm to within 1 degree Celsius of the incubator temperature. However if the test plates are stacked in piles five deep ,the center plate takes up to 4 hour to reach the same temperatures.

(vii) Incubation time

Since the position of the zone of inhibition is determined within the first few hours of incubation, the zones of inhibition may be measured as soon as microbial growth can be seen. With most of the common bacterial pathogens, definite zones of inhibition can be observed within 5 to 6 hr after inoculation.

(viii) Timing of drug application

For susceptibility testing, the agar medium is inoculated and then allowed to dry for a defined period of time before the disks are applied. This drying step is essential to

prevent leaching of the antimicrobial agent from the disk into the layer of moisture that may be left immediately after inoculation of the agar medium.

(ix) Concentration of antimicrobial agents in the reservoir

Fundamental to all agar diffusion procedures is the relationship between the size of the zone of inhibition and the potency of the antimicrobial agent. For bioassay procedures, standard drug solutions are tested on each assay plate and drug concentrations (log 10 scale) was plotted against the zone diameter (arithmetic scale). For most practical purposes, dose-response curves as essentially straight lines are being considered.

(x) **Presence of serum proteins**

Many antimicrobial agents are reversibly bound to serum proteins. Agar diffusion susceptibility tests are performed in the absence of serum proteins, and protein binding is a consideration only when attempting to translate the results into terms that would predict responsiveness of an individual patient (Victor Lorian 2005).

IMAGE ANALYSIS

Image analysis is the extraction of meaningful information from images, mainly from digital images by means of digital image processing techniques. Image analysis task can be symbol as reading bar coded tags or as sophisticated as identified a person from their face.

Computers are indispensible for analysis of large amounts of data, for task that required complex computerization, or for the extraction of quantitative information. On the other hand, the manual visual cortex is an excellent image analysis apparatus, especially for extracting higher level information, and for many applications- including medicine, security, remote sensing.

Computer image analysis largely contained for the field of computer or machine vision, and medical imaging, and makes heavy use of pattern recognition, digital geometry and signal processing. This field of computer science developed in the 1950s at academic institutions such as the originally as a branch of artificial intelligence and robotics.

Standard light or phrase-contrast microscopy, combined with specific staining techniques (such as the gram stain), is the most rapid method of establishing the presence and making the initial identification of most bacteria. Discernment of subcellular structure, however, requires the use of electron microscopy. Tranmission electron microscopy, in which electrons pass through ultrathin sections of the specimen, is used to observe internal structures, such as ribosomes, and layers of the cell envelop. Scanning electron microscopy, in which electrons are scattered off the surface of metal coated specimens, is used to observe details of the cell surface.

Reversal of image contrast when a positive print is made from a photographic negative represents, perhaps, the simplest and most practiced form of image processing. More sophisticated techniques were developed years to enhance images of the moon and other celestial objects the first to develop processing techniques to study the structures of biological specimens imaged by electron microscopy.

Humans are visual creatures. For example, we describe beauty in visual terms. We categorize visual features of importance to us. For example, we compared an object's size with the size of a standard. We also compared the shape of an object with other familiar shapes. Image analysis attempts to formalize this type of process by the use of an image analyzer

An image analyzer comprises an image acquisition device, a means for converting the image to digital form, and software/hardware to process the data in order to extract

the desired information from it.

But conventional or classical image analyzers have serious fundamental problems in the image features that they claim to measure. They usually merely count pixels. They are a product of a more or less cottage industry of numerous independent investigators who have, over a long period of time, introduced many an ‘image feature’ that purports to represent some characteristic of the image of an object. Unfortunately, most of these features are unreliable guides to the fundamental characteristics of the image of an object.

The terms image analysis and image processing are often incorrectly assumed to be synonymous, but they refer to different aspects of the treatment of image data. Image analysis involved the quantification and classification of images and images and objects of interest with in images. Image processing refers to any technique, which alters, and displays, in more tangible form, the information contained in images.

Thus an image analysis system is a tool for gathering data from an image. Image analysis methodology used to extract data from images rather than counting or measuring directly. The advantages are that the captured images are permanent, allowing more time for study, and they can be subjected to enhancement and automated analysis techniques to improve data quality.

Image processing extends the electron microscopist’s ability to study imaged biological structure because details that may be invisible to the naked eye can be clearly revealed. An obvious benefit of the clearer images and structural information is an enhanced understanding of biological structure-function relationships. Correlation with x-ray diffraction, biochemical, genetic, immunological, and model building studies makes image processing a powerful tool for investigating the basis of molecular events in living systems, particularly in real time by non-invasive methods.

Elements of Visual Interpretation

Recognizing targets is the key to interpretation and information extraction. Observing the differences between targets and their backgrounds involves comparing different targets based on any, or all, of the visual elements of tone, shape, size, pattern, texture, shadow and association.

Tone refers to the relative brightness or colour of objects in an image. Generally, tone is the fundamental element for distinguishing between different targets or features. Variations in tone also allow the elements of shape, texture, and pattern of objects to be distinguished. Shape refers to the general form, structure, or outline of individual objects. Shape can be a very distinctive clue for interpretation. Size of objects in an image is a function of scale. It is important to assess the size of a target relative to other objects in an scene, as well as the absolute size, to aid in the interpretation of that target. A quick approximation of target size can direct interpretation to an appropriate result more quickly. Pattern refers to the spatial arrangement visibly discernible objects. Typically an orderly repetition of similar tones and textures will produce a distinctive and ultimately recognizable pattern.

Digidoc, Digimizer, and Macro Auto System

These are three image analyzing system. AlphaEase FC digidoc software includes tools to optimize the image display by adjusting contrast automatically or manually. Hard-to-see portions of the image can be clarified by converting the image from positive to negative, using digital filters, or applying a false color map. Notes, labels, arrows, lines and other drawing tools can be recorded directly on the image using the annotation function available in AlphaEaseFC software. Annotations are superimposed on the image when a hard copy is printed and can either be saved as a template file or as part of the image.

AlphaEase FC software also included a broad array of analysis tools, including molecular weight calculation, Rf determination, I-D lane densitometry, 2-D spot densitometry, quantitative PCR, microtiter plate reading, object distance measuring, gel scoring, and automatic colony counting.

Applications of alphadigidoc

Molecular weight determination

This opens a set of tools for entering the values of known molecular weight markets and determining the molecular weights of unknown bands on the image.

Calculating R_f values

To obtain accurate R_f values, specify the location of the wells.Once the origin and dye from have been defined, they are used to calculate the R_f values of any bands that are added.

ID Multi (Line Densitometry)

Access a set of densitometry tools with which bands on a gel can be scanned and quantities in a lane format. There are two different ways in which this can be done, Auto lane and Auto grid. Auto gird allows the user to manually define the lane number, lane shape, and scan width of the Grid. Auto lane is a completely automated feature which will automatically define lane number, and band finding parameters for the user.

Spot density tools

Opens a set of tools with which the density of bands, spots or other objects can be measured. A two dimensional area of interest (or object) is created and the density is obtained through the corresponding pixel intensity values designated as IDV.

Spot Density measurements

As objects are drawn, their density data is automatically calculated and displayed in a data window. Any time an object is drawn or detected, the data in the window are updated.

Data definitions

is the number assigned to each object on the image in the order in which they were drawn. This object number is also shown in the corner of the object.

IDV is the sum of all the pixel values after background correction

IDV = Σ (each pixel value – BACK)

% is the percentage that each box, ellipse, or freehand drawing contributes to the total density measured thus far, taking background correction into consideration. The sum of the values in this column will be 100.

AREA s the size (in pixels) of the region enclosed by the box, ellipse, or freehand drawing AVG is the average value (after background correction) of the pixels enclosed.

AVG = IDV ÷ AREA

BACK is the background value that will be subtracted from all the pixels in the object.

The ruler function

Before any measurements can be obtained, a standard scale must be established. The scale that is created will serve as the standard for all measurements. Therefore, it is necessary to draw the scale line according to some standard measurement within the image.

In case of macro auto calculation system a program is used to analyzing an

existing image on the computer. On the program when we are giving the file name of a particular image the software automatically finding out the image and analyzing it through different overlays steps.

Parameters of digimizer

Digimizer have the following parameters

1. Area
2. Length
3. Radius
4. Perimeter
5. Intensity
6. Units
7. Minimum area
8. Maximum area

Pixel

The term “pixel” is actually short for “Picture Element”. These small little dots are what make up the images on computer displays, whether they are flat-screen (LCD) or tube (CRT) monitors. The screen is divided up into a matrix of thousands or even millions of pixels. Typically we cannot see the individual pixels, because they are so small. This is a good thing, because most people prefer to look at smooth, clear images rather than blocky, “pixilated” ones. However, if we set the monitor to a low resolution, such as 640 x 480 and look closely at the screen, we can able to see the individual pixels.

ADVANTAGES OF IMAGE ANALYSIS ON PETRI PLATE EVALUATION

- Digimizer automated method is very rapid. By a single click we can get a lot of information regarding with the plates and zones.
- Digimizer values are more accurate, than normal conventional values because here the measurement is done by the computer with the help of software. In clinical field, if we are giving some particular barcode to particular plate, full information regarding with that plate will go directly to the patient data sheet.
- PAE studies can be done on agar plate by the help of density values.
- Different regional wise susceptibility studies can be easily done by the help of image analyzing process
- Computer can give the values in whatever be the unit we needed i.e., whether it is pixel, cm, or mm etc.
- We can measures the combined drug action on a Petri, plate, whether it is synergic or antagonistic or mere additives because computer can recognize whatever be the shape of the zone.
- By checking the intensity or density of different area we can tell how uniform the swabbing is.
- The zone is affected by many factors like depth of the media, bacterial population density etc, if we are taking the value in terms of density and minimizing the zone density from the growth density the errors due to different bacterial population and depth of the agars can be minimized.
- By the time zone analysis of Petri plate we can get the growth curve and from that we can identify the doubling time.
- By normal conventional way of measurement we can get only diameter, but here we can are, perimeter, radius, average intensity, I.D.V, Avg Density etc by a single click.
- Here the result is reproducible as the calculations are done by the help of image

analysis.

- As outsourcing is becoming familiar now days in every field, the different research teams or organization of different parts of world can correlate their research result properly through image analysis.
- If any different colour density is accruing due to contamination, computer can express it by mean of some digital values.
- Even though if there is any irregularity in zones due to improper diffusion or irregularities of disk or well happen computer can analyze and measure the required area.
- If very advanced high mega pixel camera or an instrument attached with advanced microscope, we can get an idea about the organism present in the images.

OBJECTIVES

To perform complicated image processing functions to bring out relevant features that may provide very useful information but are difficult to extract by conventional means.

- To develop a method to study antimicrobial susceptibility through automated image analysis.
- To perform the test in a relatively short duration of incubation time for antimicrobial susceptibility studies to facilitate an early initiation of treatment
- To study the rate of growth of organisms on agar plate and to crosscheck the class of organism by doubling time determination, performed along with antimicrobial susceptibility test.

REVIEW OF LITERATURE

IMAGE ANALYSIS ON PETRI PLATE EVALUATIONS

M. Kolbert et al. (2004) compared the measurement of inhibition zones by the automated OSIRIS system with manual measurement. Variations of +3 mm in zone size measurements were defined as tolerable. Very major errors (i.e., classification of a resistant isolate as susceptible by the OSIRIS system) occurred in <1% o tests).It was concluded that the OSIRIS system was a rapid and reliable system for measuring disk susceptibility test results on round and square agar plates.

A. Nijis et al. (2003) found the OSIRIS and SIRISCAN 2000 systems are two semi automated systems that can be sued to read and interpret the results on disk diffusion agar plates. He compared both systems versus the NCCLS standard method of visual reading with a ruler. The results obtained with both systems in comparison with those obtained by the classical non automated means of interpretation were excellent, with correlation coefficients of 0.96 for both systems. The overall agreements for susceptibility interpretation were 96.56 and 96.24% with the OSIRIS and SIRISCAN systems, respectively. Overall, both the OSIRIS system and the SIRISCAN systems are comparable and reliable systems for determination of interpretative categories from the zone diameters of standard disk diffusion test plates.

J.M. Andrews et al. (2000) demonstrated the Oxoid Aura image antibiotic sensitivity test system, used as a stand-alone device. He compared with manual zone measurement and use of a template, for the determination of sensitivities. An overall correlation coefficient of 0.99 was observed for zone diameters measured using the Aura image system and zones measured manually, when the differences between zones were within 3 mm. 5.4% of zones showed a difference in zone diameter between manual and automated measurement of >3 mm.

E. Kent Korgenski et al. (1998) investigated the BIOMIC system includes software and a video-assisted plate reader that functions with a personal computer to automate, speed read, and interpret standard antibiotic disk diffusion test plates. The video reader helps standardize endpoints, speeds quantitative measurements by 40 to 90%, and reduces fatigue and transcription and interpretation errors. He conclude that the video-assisted plate reader is a reliable system for determining interpretative categories from zone diameters of standard antibiotic disk diffusion test plates.

Schoevers E.J et al. (1992) developed a computerized image analysis system (IAS) for reading the agar diffusion test automatically. The inhibition zones were measured by hand and by the IAS directly from the bioassay plate. Both methods were positively correlated. It is concluded that the IAS methods is an objective and accurate alternative for reading the agar diffusion test.

Simon Johns (2003) found single radial immuno diffusion (SRD) is a simple yet powerful technique that is routinely sued in many clinical laboratories for a side variety of analyses. Despite its simplicity, the technique as currently practiced suffers from a major drawback due tot eh fact that in general, the measurement of the reaction zones generated by the assay is performed manually. This is a task that is time-consuming and error-prone. To overcome these problems, a novel method of automating inhibition zone measurement has been developed. The new system has been extensively tested and compared with the standard, manual method. He reports the performance of the new method in the assay of the potency of influenza vaccines.

Karren Capper (2003) found one of the main functions of microbiology laboratories in modern pharmaceutical companies is to carry out routine quality control testing. Modern image handling and archiving systems can dramatically improve the reliability of microbiology QC systems and ensure speedy transmission of potentially vital data to the appropriate site.

APPLICATIONS OF IMAGE ANALYSIS

Michael Putman et al. (2005) determined bacterial colony counting is a significant technical hurdle for vaccine studies as well as various microbiological studies. He show that an automated colony counter can process images obtained with a digital camera or document scanner and that any laboratory can efficiently have bacterial colonies enumerated by sending the images to a laboratory with a colony counter via internet.

Feng Chen et al. (2001) found a novel nucleic acid stain, SYBR Gold, was used to stain marine viral particles in various types of samples. Viral particles stained with SYBR gold yielded bright and stable fluorescent signals that could be detected by a cooled charge-coupled device camera or by flow cytometry. Estimates of viral concentration based on digitized images were 1.3 times higher than those based on direct counting by epifluorescence microscopy. The potential application of digital image analysis and flow cytometry for rapid and accurate measurement of viral abundance in aquatic environments is discussed.

Corine M. et al. present new software (Root flow RT) for measuring the expansion profile of a growing root at high spatial and temporal resolution. The software implements an image processing algorithm using a novel combination optical flow methods for deformable motion. The algorithm operates on a stack of nine images with a given time interval between each (usually 10s) and quantifies velocity confidently at most pixels of the image.

Holger Daims et al. (2006) found the combination of microscope and molecular techniques to detect, identify and characterize microorganisms in environmental and medical samples are widely used in microbial ecology and biofilm research. He introduces daime (digital image analysis in microbial ecology), a new computer program integrating 2-D and 3-D image analysis. This image analysis method complements

recent molecular techniques for analyzing structure-function relationships in microbial communities and will help to characterize symbiotic interactions among microorganisms.

MODIFICATION AND EVALUATION OF ANITMICROBIAL SUSCEPTIBILITY METHODS

Harold J et al. (1970) modified agar-diffusion technique for micro bioassay of antimicrobial agents to increase sensitivity of the technique and to extend the range of antimicrobial agents to which it sis applicable. This micro technique requires only 0.02 ml of an unknown test sample for assay, and is capable of measuring minute concentrations of antibiotics in buffer, serum, and urine.

Arthur L et al. (1979) performed standard Bauer-Kirby disk tests. Each disk test was observed and zone diameters were measured under two lighting conditions (transmitted light and reflected light). The two lighting systems produced similar zone measurements (+2 mm) with 96% of the tests).

Antal Kabay (1971) described a rapid quantitative assay by the cylinder plate diffusion method, in which *Bacillus Stearothermophilus* ATCC 12980 was used as the test organism. After 4 hr of incubation at 65°C, the zones of inhibition can be read off easily with the naked eye. The 4 hr assay was compared under identical conditions with the conventional 16 to 18 hr agar diffusion assay. No difference in accuracy between the two methods was detected. In both cases, the coefficient of variation for replicate tests was less than 2%.

John V Bennett et al. (1965) modified the assay with a specially designed agar punch, it is possible to prepare the small agar wells very quickly. The saving in serum resulting from fewer replications of standards with the large plates, and the small

volume of the agar wells, make it economically feasible to use pooled human serum for the standard antibiotic solutions. With this method, it has been possible to test large numbers of clinical specimens in a minimal time, and with accuracy consistently better than 10%.

Bauer et al. ((1966) demonstrated an antibiotic assay in which the bacterial broth suspension is streaked. Surplus suspension is removed from the swab. After the inoculum has dried. Plates are incubated within 30 min. After overnight incubation, the zone diameters (including the 6mm, disk) are measured with a ruler. The zone diameters are a recorded and interpreted.

Davis and et al. (1971) investigated of the several factors cause variation in zone diameter. Of these factors the most serious is the unequal exposure of the individual plates at top or bottom of several to temperatures above and below room temperature. A major variable is the varying time interval between pouring seeded agar and the time of applying the pads with antibiotic to the plates. This influence of time of setting and the effects of several other sequential operations are combined into a composite variable. Such factors as volume of agar poured, wedge shape of agar in a dish, volumetric errors in dilutions, and timing considerations are studied.

APPARATUS AND MATERIALS

Apparatus

Conical Flask (250 , 500 ml)	:	Borosil
Sterile Swab	:	Himedia
Test tube	:	Borosil
Petri dishes	:	SD Fire-chem LTD
Non absorbent cotton	:	Jay Cot Industries
Micropipette	:	Varipipettes
Microtips	:	Tarsons
STD flask (10ml, 100ml)	:	Borosil
Measuring cylinder	:	Borosil
Zone scale	:	Himedia
Heating mandle	:	Guna enterprises

Instruments

Hot air oven	:	Technico equipment
Autoclave	:	Universal autoclave
Horizontal laminar flow unit	:	Clear air instrument Inc
Incubator	:	Technico incubator
Image capturing system	:	Alpha DigiDoc RT
Image analyzing system	:	Alpha DigiDoc RT, Digmizer,Macroauto system
Compound microscope	:	Motic
Refrigerator	:	Kelvinator
Electronic balance	:	Shimadzu

DRUGS USED

Ciprofloxacin injection (2mg/ml)	:	Cipla
Amikacin Injection (2mg/100ml)	:	Ikon Remedies Pvt. Ltd

MEDIA USED

Muller-Hinton Agar Media

Muller-Hinton broth gelled by the addition of 2% agar (bacteriological grade).

Ingredients

Casein acid hydrolysate	:	17.5gm/Lt
Beef infusion	:	300 gm/Lt
Soluble Starch	:	1.5 gm/Lt
Final p^H at 25^{o}C	:	7.4$\pm$0.2

Preparation

The Muller – Hinton broth with 2% agar were dissolved in distilled water with the

aid of heat; pH was adjusted to 7.2 -7.6 using alkali or dilute acid.

Sterilization

The conical flask which containing Muller-Hinton agar was sealed with non-absorbent cotton cover and tied with paper . It was then autoclaved at a pressure of 15 psi (121^{o}c) or not less than 15 min.

Organisms used

Staphylococcus aureus NCIM 2706 was collected from national Chemical Laboratory, Pune and stored in pharmaceutical Biotechnology Laboratory, College of Pharmacy, SRIPMS, Coimbatore – 44. The strains were confirmed for their purity and identity by Gram's staining method and its characteristic biochemical reactions. The selected strains were preserved by sub culturing them periodically on agar slants and storing them under frozen conditions. For the study, fresh 24 hrs, broth culture was used.

Working conditions

The working area is cleaned with acetone. Before commencements of the work, the working areas are sterilized using UV light. The entire work was done using horizontal laminar flow hood so as to provide aseptic conditions. On incubation it was checked for the growth of microorganisms and absence of growth confirmed aseptic working conditions.

METHODOLOGY

Determination of antibiotic activity using agar well technique

Muller-Hinton agar plates were prepared in asceptic condition. The plates were allowed to solidify and inverted to prevent the condensate failing on the agar surface. The plates were dried at 37oC before inoculation. The organisms were inoculated in the plates prepared earlier, by dipping sterile swab in previously prepared inoculums, removing the excess of inoculums by pressing and rotating the swab firmly against the sides of the culture tube above the level of liquid and finally swabbing the swab all over the surface of the medium, rotating the plates and swab again. Finally press the swab round the edge of agar surface. It was allowed to dry at room temperature, with the lid closed. The agar well were made placed, on to which drug solutions were applied in the previously inoculated surface of the Muller-Hinton agar plate and it was kept in refriegerator for 15 min to facilitate uniform diffusion of the drug. Plates were prepared in triplicate and overnight incubation had done. Observations were made for zone of inhibition around the drugs and recorded.

The antimicrobial activity for staphylococcus aureus strains were carried out using the drugs ciprofloxacin, amikacin at different concentrations, the results were recorded. The graphs obtained from different measuring methods are compared by trend line equations

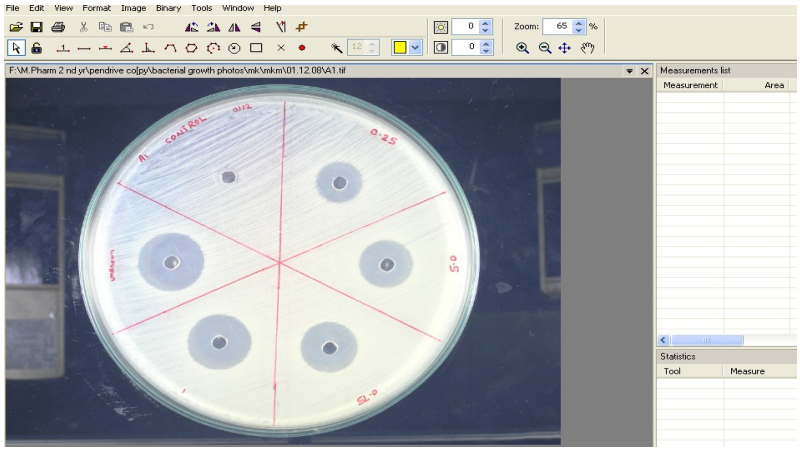
CAPTURING THE IMAGES

- Capturing the images was done by Alpha Digi Doc RT image analyzing systems.
- Kept petri plates inside the Digi Doc and adjusted the positions after switched on the camera and epi white
- Then checked the clarity by exposure view and if it was clear acquire the images.
- The exposure time was adjusted to 100 milliseconds.
- Auto contrast was given in contrast option
- In focus, it was given of autofocus
- 1024*768 resolution was selected
- 174 was kept as zoom control

METHODOLOGY FOR AUTOMATED ZONE READING SYSTEM

Software used 1: Digimizer

FIG : 2



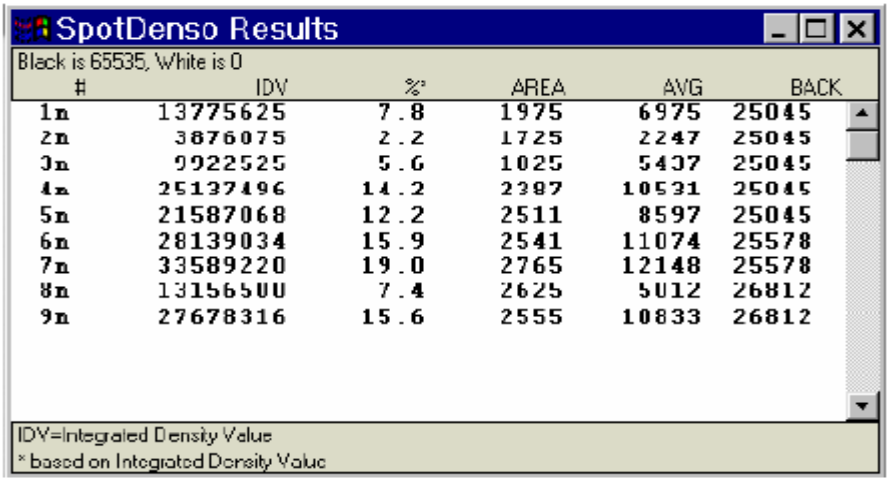
The images captured by alpha DigiDoc RT was opened in Digimizer window

- Selected & made a single click on the zone area of the images.
- Then we could see a list of information regarding with each zone, i.e., the area average intensity, length, perimeter, radius, maximum area, minimum area and unit.
- Set the mm as the unit
- The information we got by clicking on the focus area was copied to the MS- excel sheet and draw the graph we needed.

Software Used 2: Macro Auto System

- The images captured by alpha DigiDoc RT was saved into the system.
- Give the file name on the place of the program for macro calculations
- Intensity threshold of left and right and no of overlays was set
- Give the auto correction if necessary.
- Allow to run the program.
- After processing over took noted the values in terms of number of pixels.

Measuring zone of inhibition by using DigiDoc
Software used : Alpha DigiDoc



Example Of A Spot Density Data Window

FIG : 3

- The images were opened in alpha DigiDoc window.
- Inside the analyzing tool, select the spot denso tool.
- Selected the circle or ellipse option.
- On the image put a 30 mm circle which should cover the zone area with some growth field.
- By the same way put 3-4 30mm circle around each zone and find out their average.

- In spot Denso data window we can get a set of information regarding with this circles, i.e. IDV area, AVG density, back ground value etc.
- By subtracting the values of average density of 30mm zone circle from growth field density we got the different values which were indirectly showing the different concentration.
- Analyzed all zone by this ways and plot the graph.

Time zone studies for early detection of zone of inhibition

- By the conventional method prepare the agar plate, different concentration of drugs all 24 hours strain, on previous day of the experiment.
- On the day of experiment swab the plates and make the well and apply the drug.
- Images were taken in different time intervals.
- Nearly from the 8^{th} hour on wards, took the reading of the diameters from images captured.
- Plot the graph of 7, 7.30, 8, and 24^{th} hour.

Growth curve and doubling time on agar plate

- The images captured at various time intervals were opened in alpha Digi Doc window.
- Opened the spot Deso tools on the analyzing options.
- Selected the circle and put the circle on different part of the image and noted the density values from different hours images.
- Plotted the graph by taking time on x axis and density on y axis.
- Selected the linear portion of growth curve and plotted their log value graph.
- Draw trend line for this log graph. And noted the trend line equations from the ms-excel.
- From the slop of the trend line found out the generation time.

RESULTS AND DISCUSSION

RESULTS

Antimicrobial susceptibility testing had been performed through image analysis. The susceptibility of *staphylococcus aureus* to the antibiotics ciprofloxacin was studied by keeping the volume of antibiotics as 30 µl by agar well diffusion method. The images captured by alpha digidoc were used for the analyzing purpose.

The antimicrobial activity of the ciprofloxacin was compared by different zone of inhibition measuring methods- conventional zone scale values, macro auto calculation system, automated digimizer and digidoc methods. For each antibiotic -microorganism combination the experiment was performed three times. The comparison of all these different methods and plates has also been done by the r^2 values of each of their trend line equation.

A time zone study was also performed by comparing the zone of inhibition of images captured at various time intervals of incubation. Also the bacterial growth curve on an agar plate had been studied. An equation for finding out doubling time was derived, using that generation time of *staphylococcus aureus* was performed.

Program for macro auto calculations

Macro for auto calculation

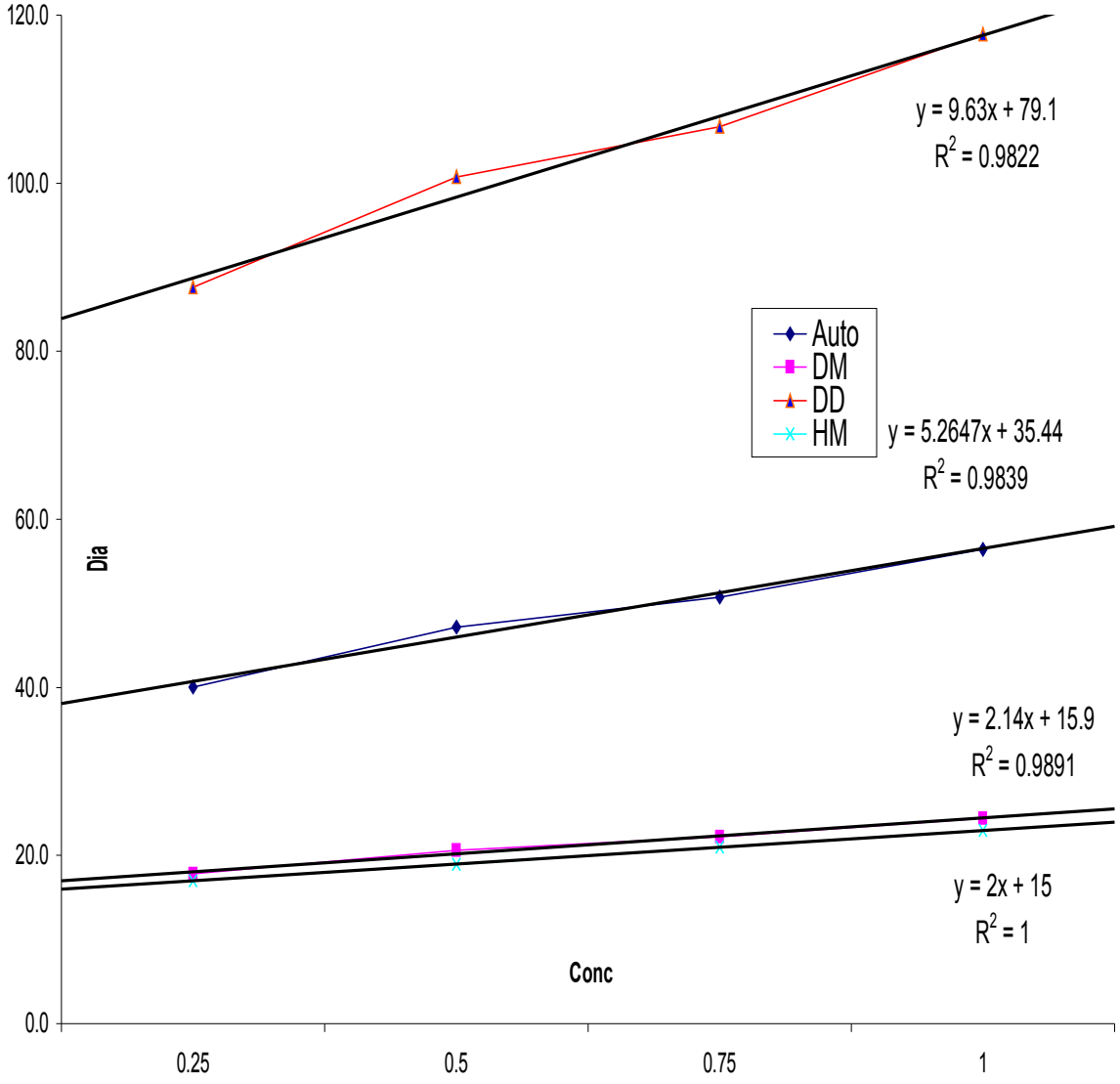
```
'#Uses "Constant.bas"
Dim App As Object
Dim Worksheet As Object
Sub Main
        Set App = CreateObject("SigmaScan.Application")
   Set Worksheet = App.GetWorksheet
   Worksheet.Show
   Worksheet.MakePermanent

        '* Turn off all measurements
        Worksheet.SetCellText("A",1, "Preparing Measurement List, One Moment Please...")
        For i=0 To NUMMEASURES-1
                App.DoNotCollectMeasurement(i)
        Next i
        Worksheet.SetCellText("A",1, "")
        App.DrawMajMinAxes=False

        '* Load the image
   ExeDirectory = App.GetExeFileDirectory()
   Dim Bacter3 As Object
   Set Bacter3 = App.OpenImage(ExeDirectory + "images\ cipro 1.tif")

   ResultCode = Bacter3.ChangeColorResolution(8, 4)
   ResultCode = Bacter3.ConvertToGrayScale
   ResultCode = Bacter3.Show
   ResultCode = Bacter3.MakePermanent
   Dim Left0(1) As Long
   Left0(0) = 175
   Dim Right1(1) As Long
   Right1(0) = 225
   ResultCode = Bacter3.IntensityThreshold(1, 1, Left0, Right1)

   Dim Left2(1) As Long
   Left2(0) = 175
   Dim Right3(1) As Long
   Right3(0) = 225
   ResultCode = Bacter3.IntensityThreshold(2, 1, Left2, Right3)

   '* Run binary (overlay) filters on the objects
   ResultCode = Bacter3.FilterOverlay(2, 2, 3, 3, 2)       '* Erode, split objects
   ResultCode = Bacter3.FilterOverlay(5, 3, 4, 1, 2)       '* Dilate everything
   ResultCode = Bacter3.FilterOverlay(6, 4, 5, 2, 2)       '* Dilate, don't merge
   ResultCode = Bacter3.FilterOverlay(10, 5, 5, 1, 2)     '* Remove edge objects
   ResultCode = Bacter3.AndOverlays(5, 1, 5)   '* Logical AND overlays 1 and 5

   '* Zoom the image an hid overlays for easier viewing
   ResultCode = Bacter3.SetZoomLevel(2.0000)
   ResultCode = Bacter3.HideOverlay(1)
```

```
    ResultCode = Bacter3.HideOverlay(2)
    ResultCode = Bacter3.HideOverlay(3)
    ResultCode = Bacter3.HideOverlay(4)

    '* Measure the objects

            '* Count the objects
            App.CollectMeasurement(M_NUMOBJECTS, "B")
    ResultCode = Bacter3.CountObjects(5)
    NumItems = Worksheet.GetCellValue("B",1)
'    MsgBox("# Objects = " +  CStr(NumItems) + " / " + CStr(ResultCode)+" \ "+CStr(M_NUMOBJECTS))
'    Exit Sub

            '* For each object find its area and shape factor
            App.DoNotCollectMeasurement(M_NUMOBJECTS)
            App.CollectMeasurement(M_SHAPEFACTOR, "A")
            App.CollectMeasurement(M_AREA, "C")
          ResultCode = Bacter3.MeasureObjects(5)

            '* Eliminate all objects not sufficiently round
            MsgBox("Removing all non-compliant objects.  Click OK to continue.")
            iNum=0
    For ii = 1 To NumItems
            Shape = Worksheet.GetCellValue("A",ii)
            ObjArea = Worksheet.GetCellValue("C",ii)
            If (Shape < 0.15) Or (ObjArea < 1500) Then
                    '* Eliminate the object
                    ResultCode = Bacter3.EliminateObject(ii)
                    iNum=iNum+1
            End If
    Next ii
    MsgBox(iNum+" objects removed.  Click OK to finish.")

    'Recorded Macro Ends
End Sub
```

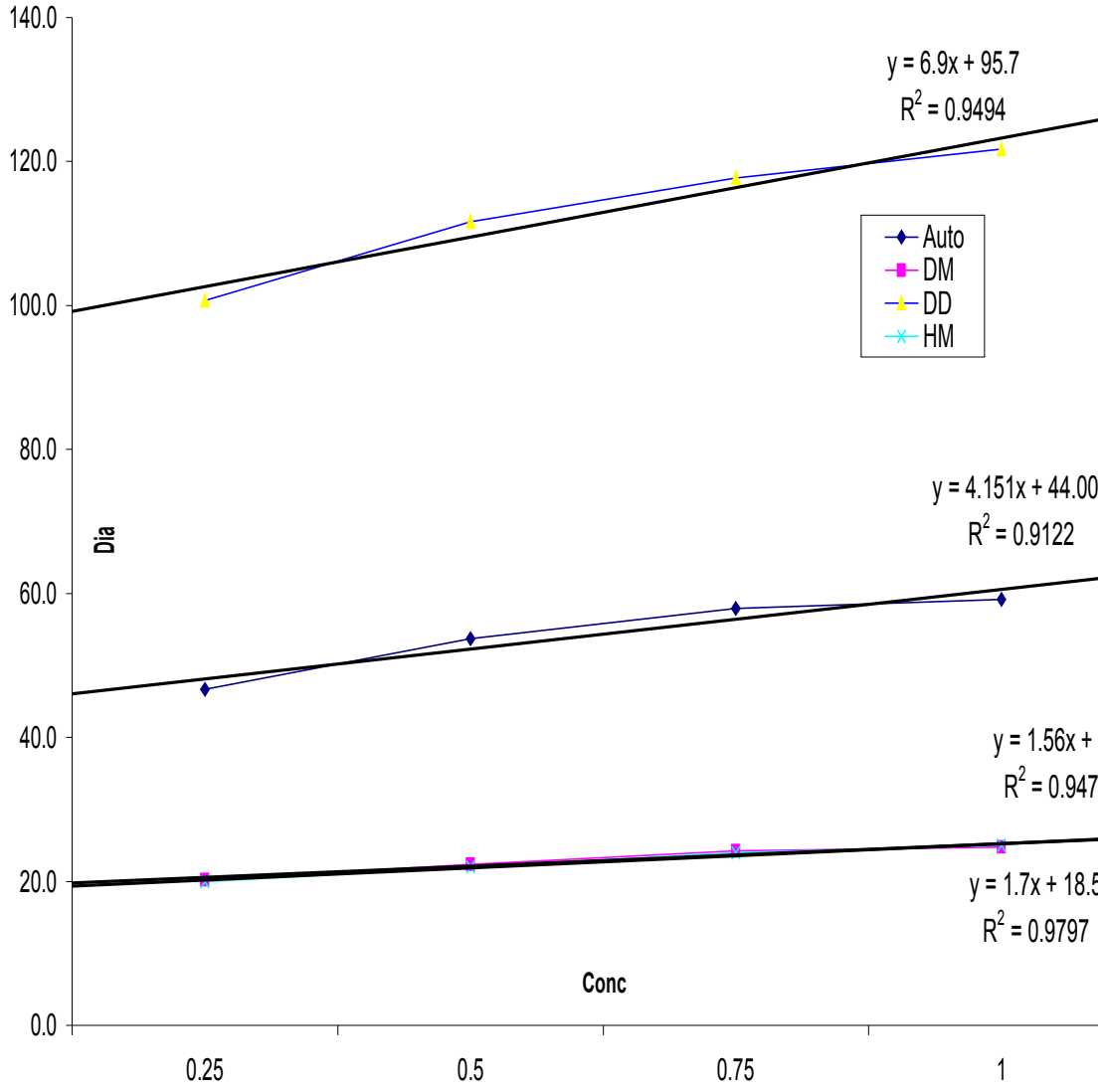
Table no : 1

Table no : 2

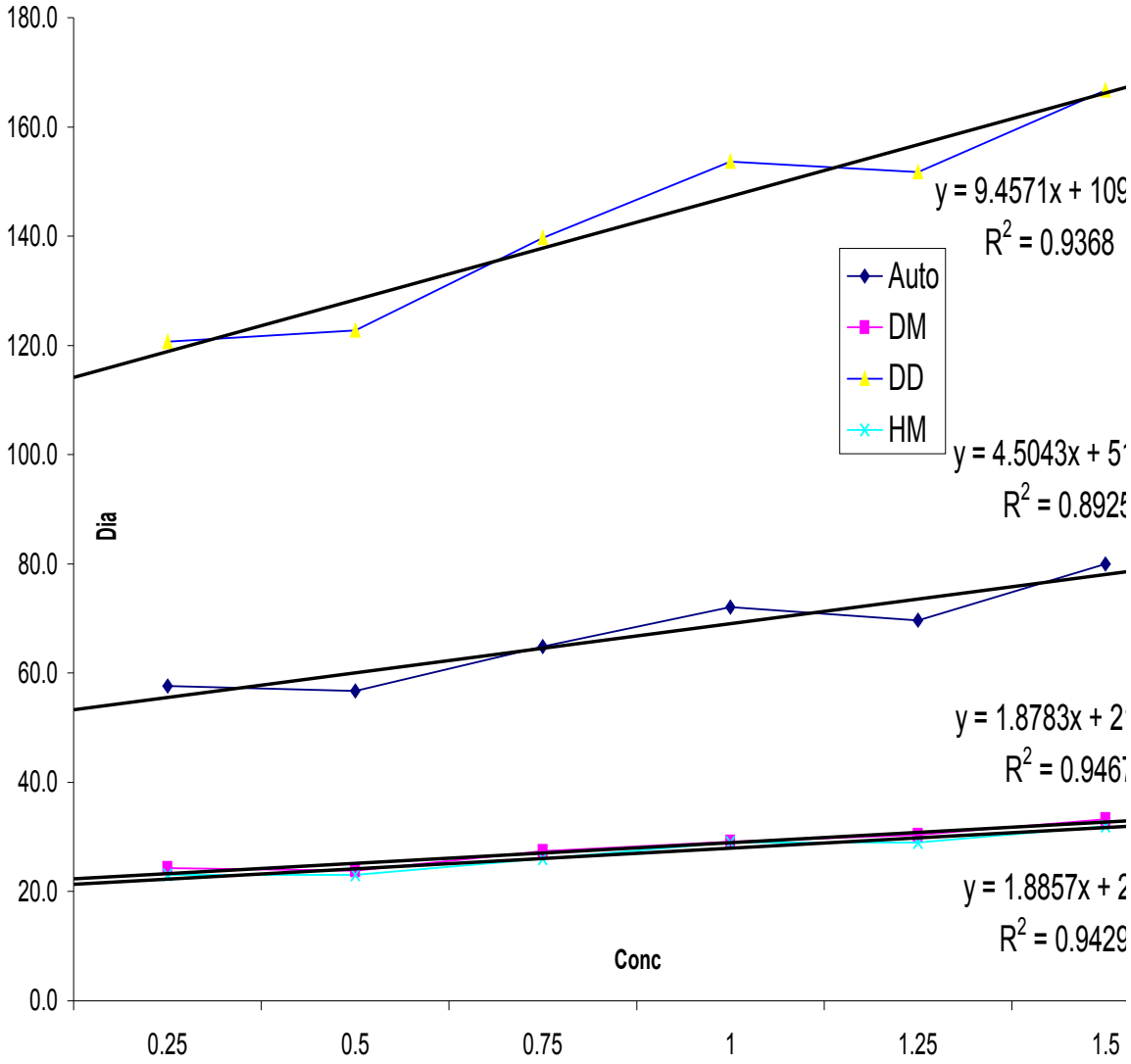
graph no:1 Cipro 1



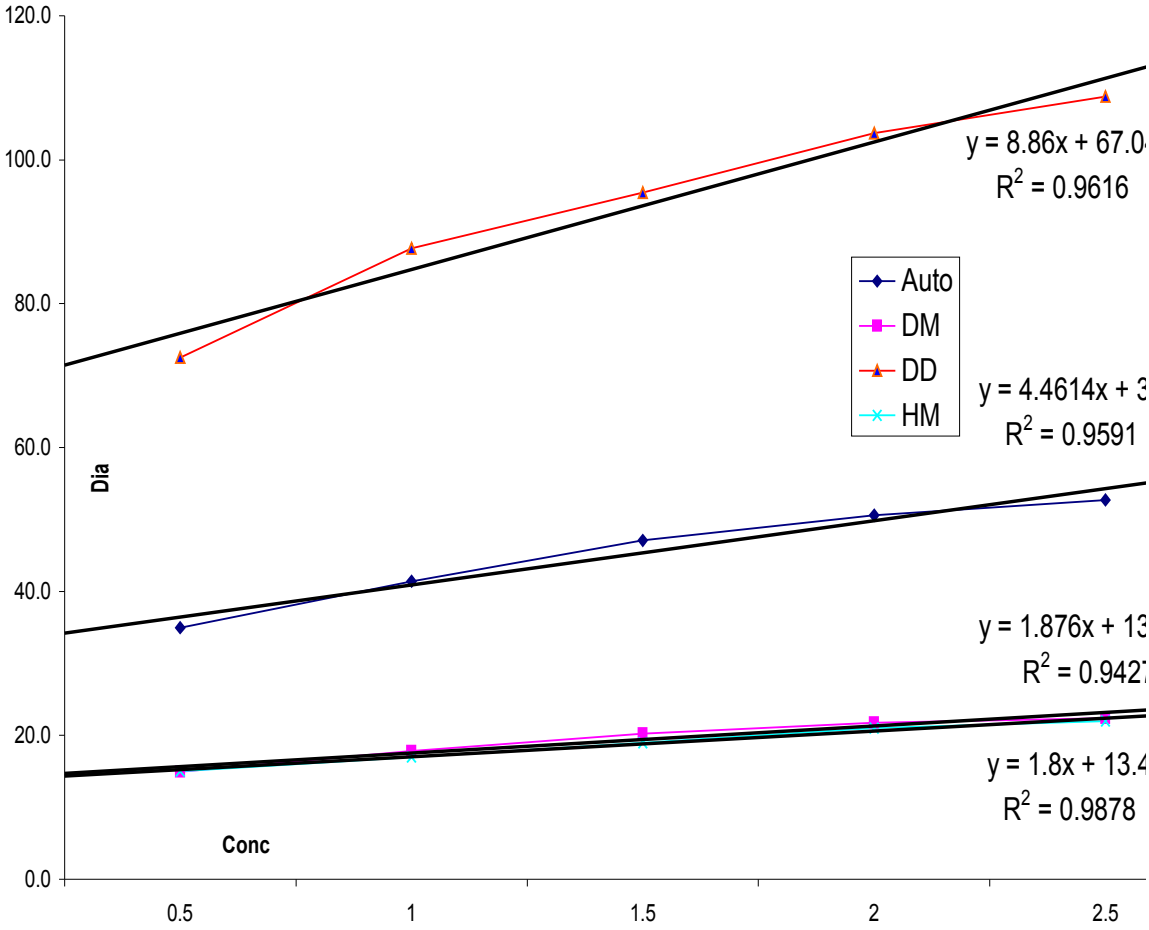
graph no :2Ciprofloxacin 2



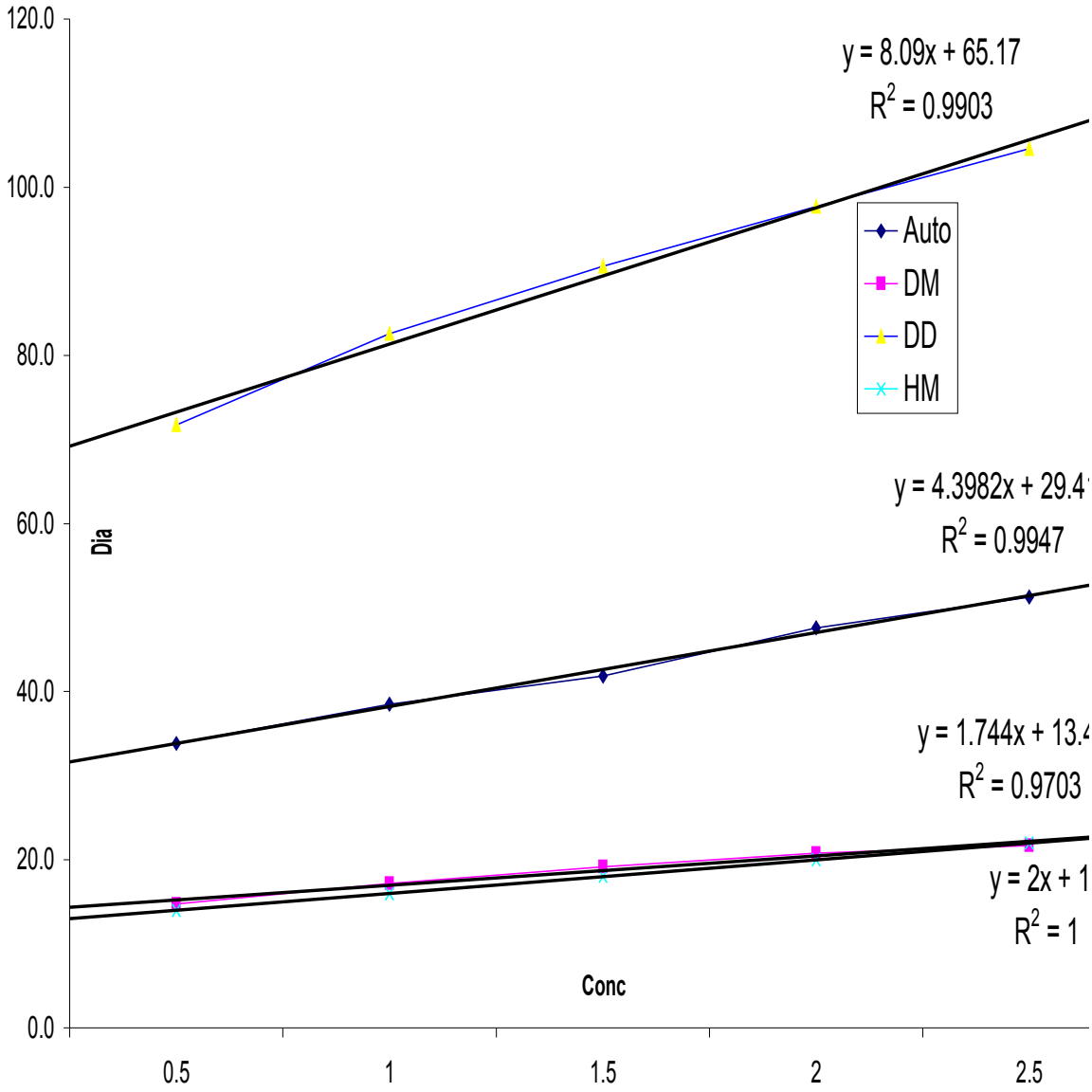
Graph No. 3: Ciprofloxacin 3



Graph No. 4 : Amikacin 1



Graph No. 5 : Amikacin 2



Graph No. 6: Amikacin 3

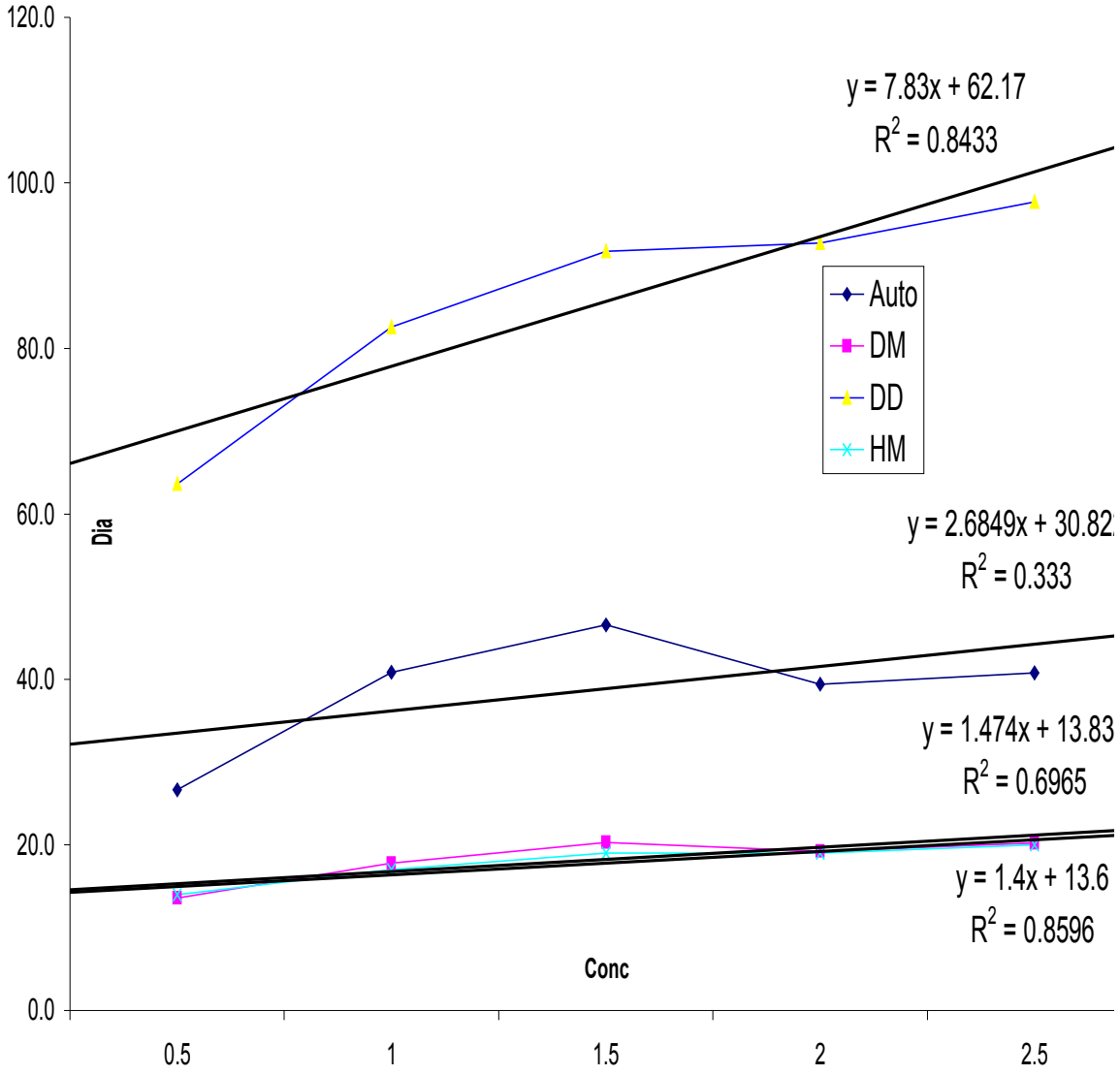


Table no: 3 Correlation Table

Serial no	Macro auto	Digimizer	DigiDoc	Himedia zone scale

1	1.7430810	1.732151	1.612879	1.554878
2	1.1539122	1.581232	1.570998	1.5
3	1.8611550	1.812789	1.825257	1.868421
4	1.0982590	1.127646	1.071841	1.125
5	0.8429700	0.898364	0.891575	0.979153

Table no: 4 Statics Matrix

	Conc.	**Macro auto**	**Digimizer**	**DigiDoc**	**Himedia zone scale**
Conc.	1				
Macro auto	0.9411259	1			
Digimizer	0.95658591	0.998	1		
DigiDoc	0.95605588	0.99	0.991	1	
Himedia zone scale	0.9069	0.9711	0.9704	0.989	1

DIFFERENT STEPS OF OVERLAYS, FIG: 4

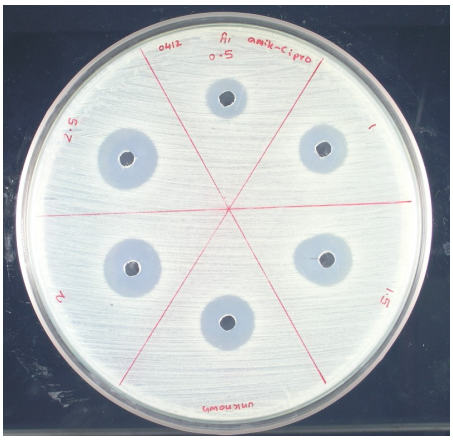


Fig: a

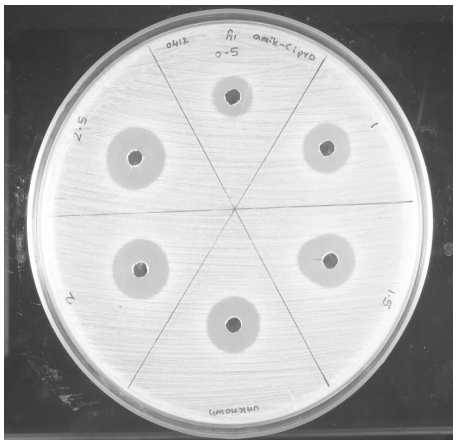


Fig: b

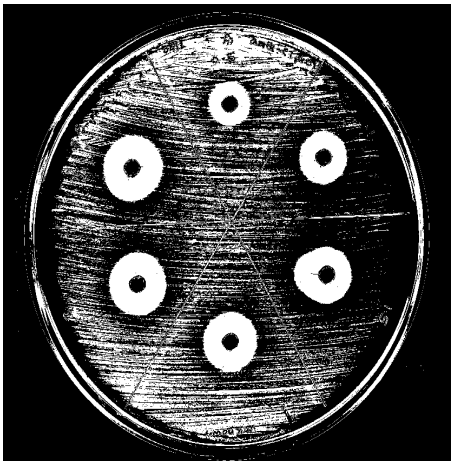


Fig: c

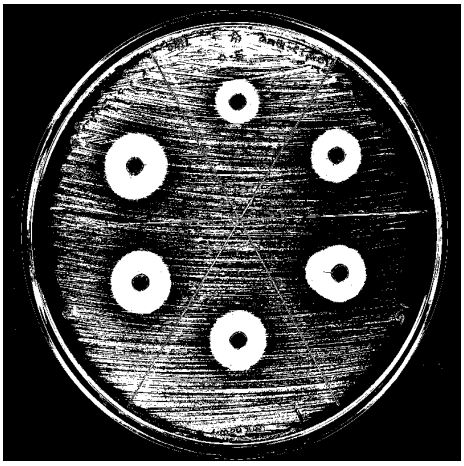


Fig: d

DIFFERENT STEPS OF OVERLAYS, Fig 4b

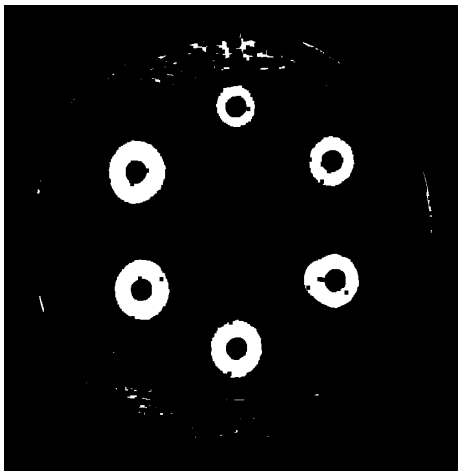
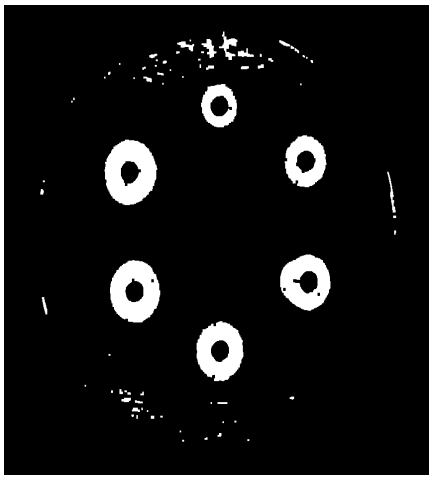
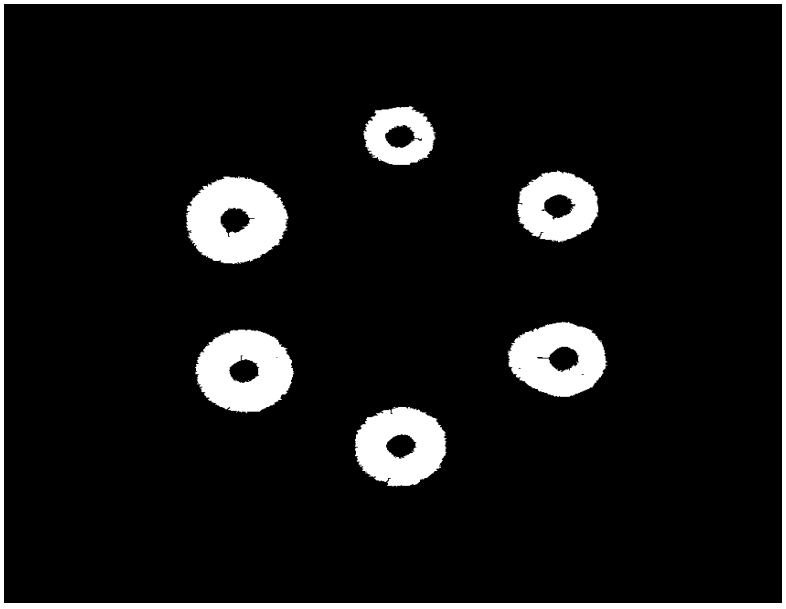


Fig: e



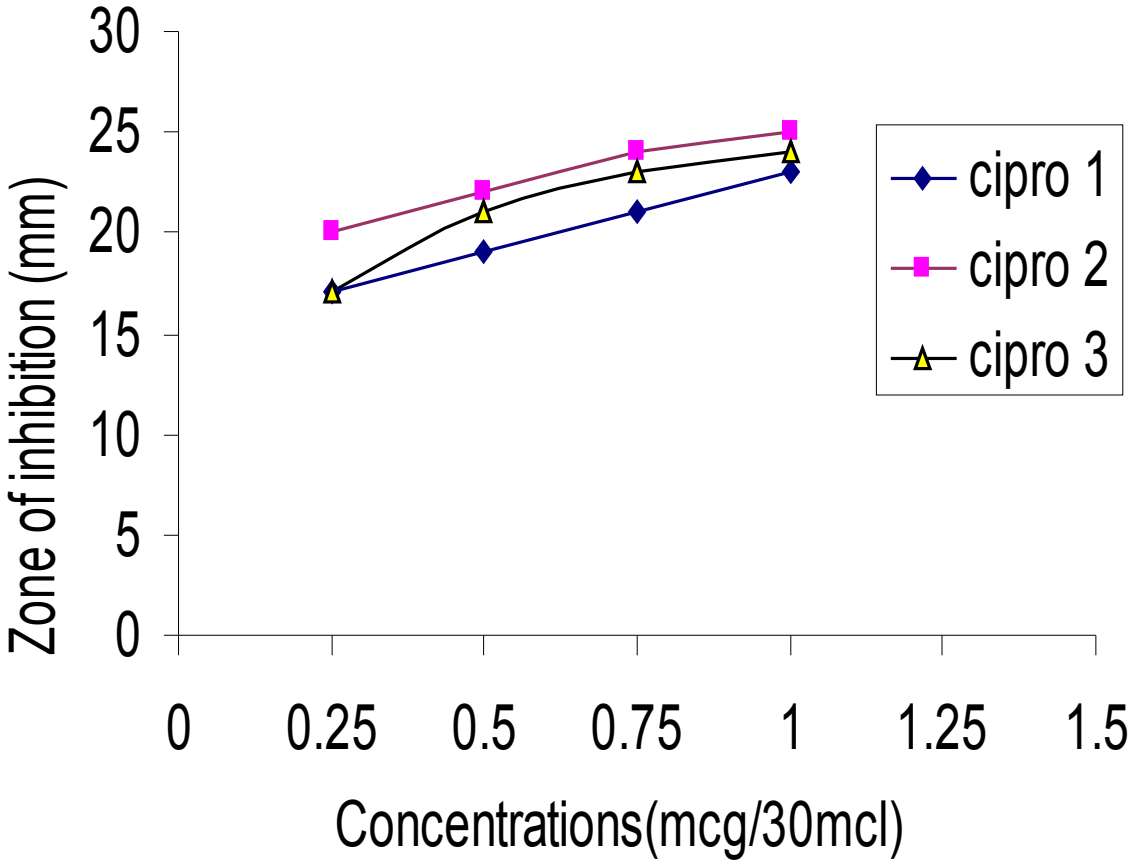
Figs: f



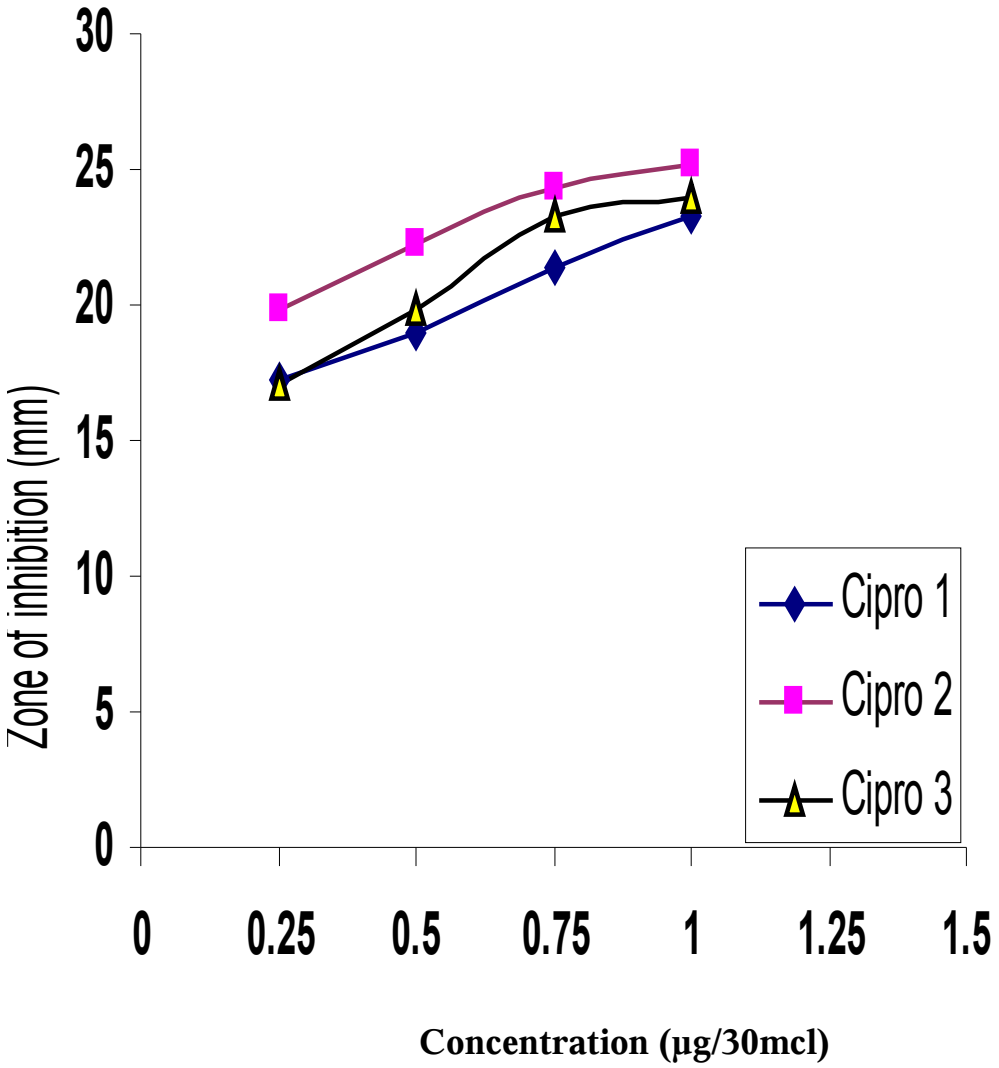
FINAL OVERLAY

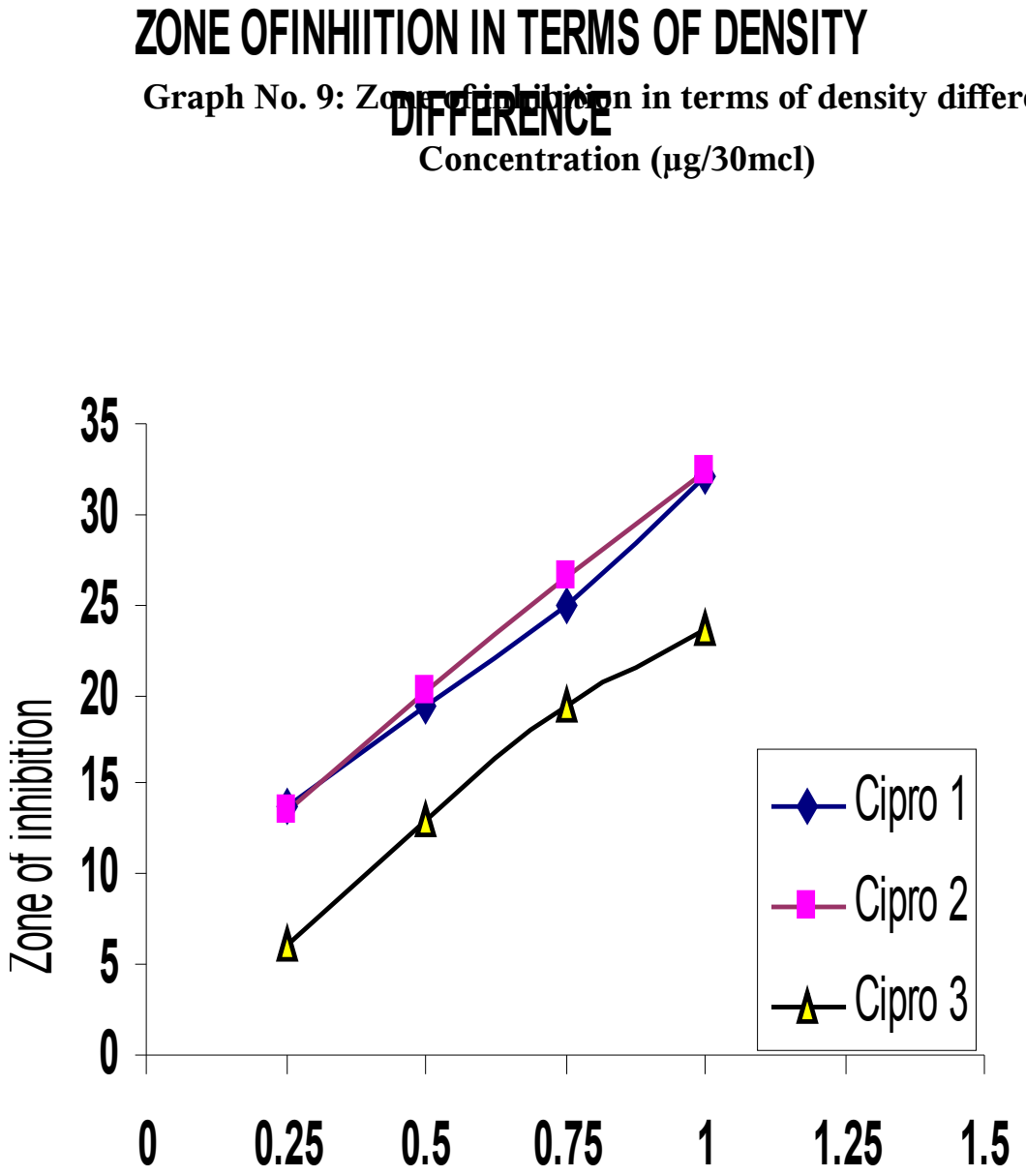
Fig: g

Graph No.7: Himedia zone scale values



Graph No. 8: Digimizer automated values



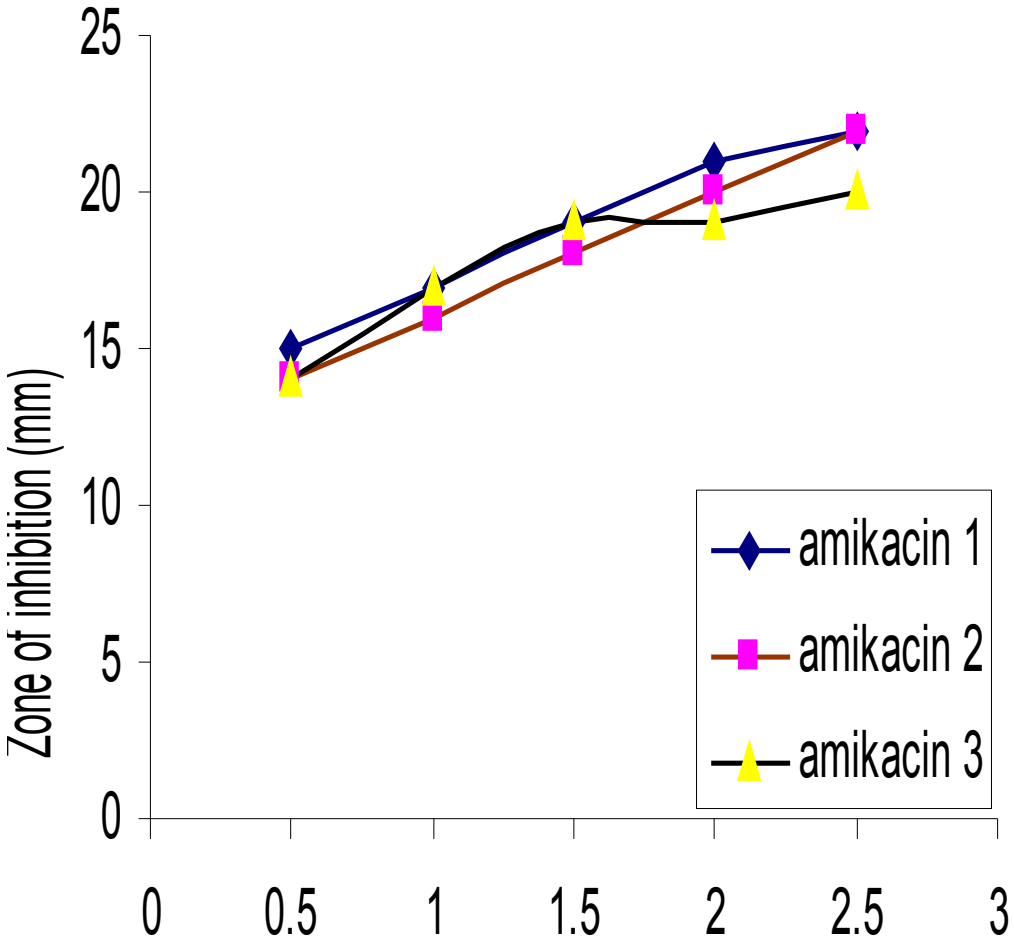


Graph No. 9: Zone of inhibition in terms of density difference

Concentration (µg/30mcl)

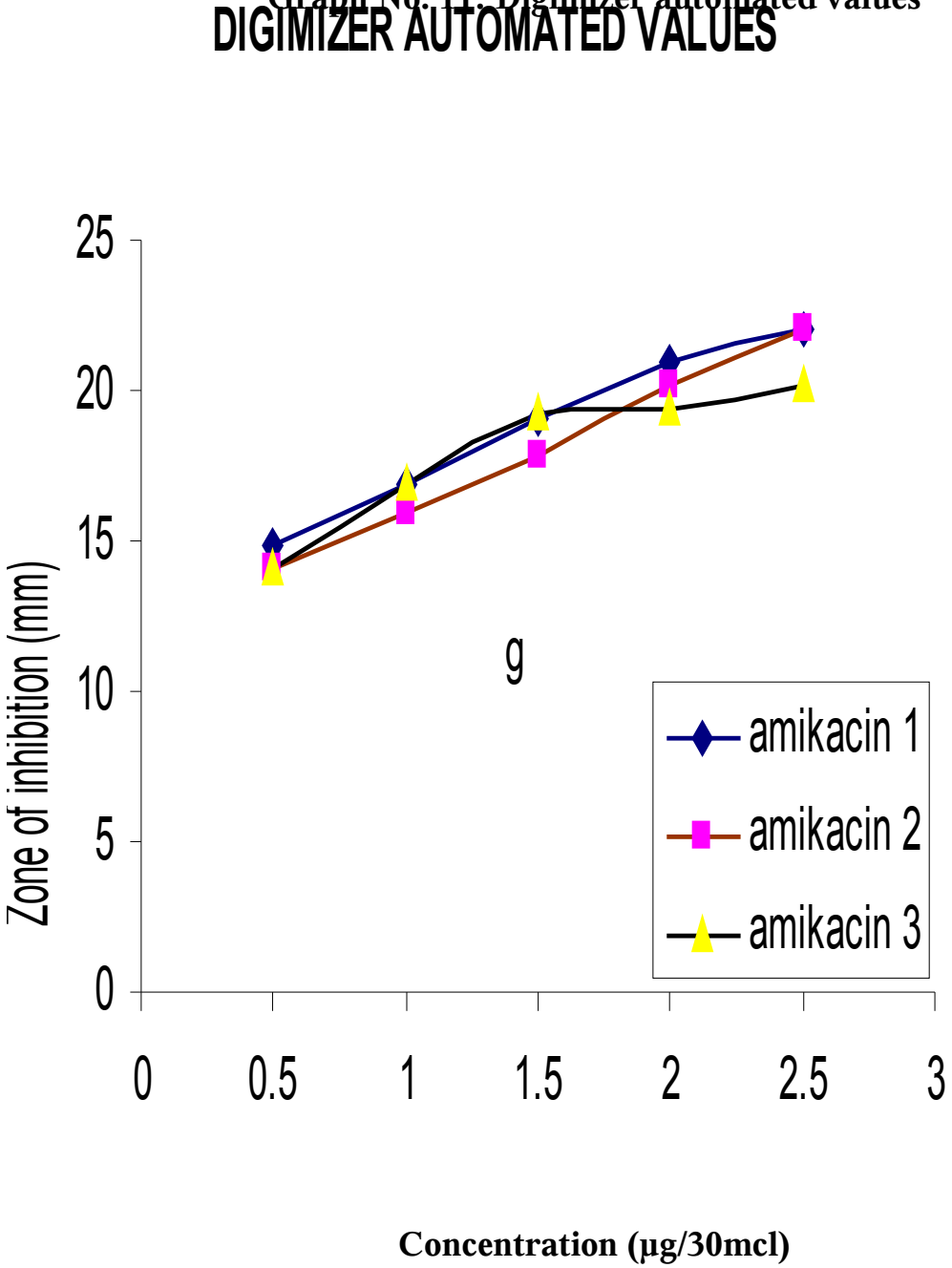
HIMEDIA ZONE SCALE VALUES

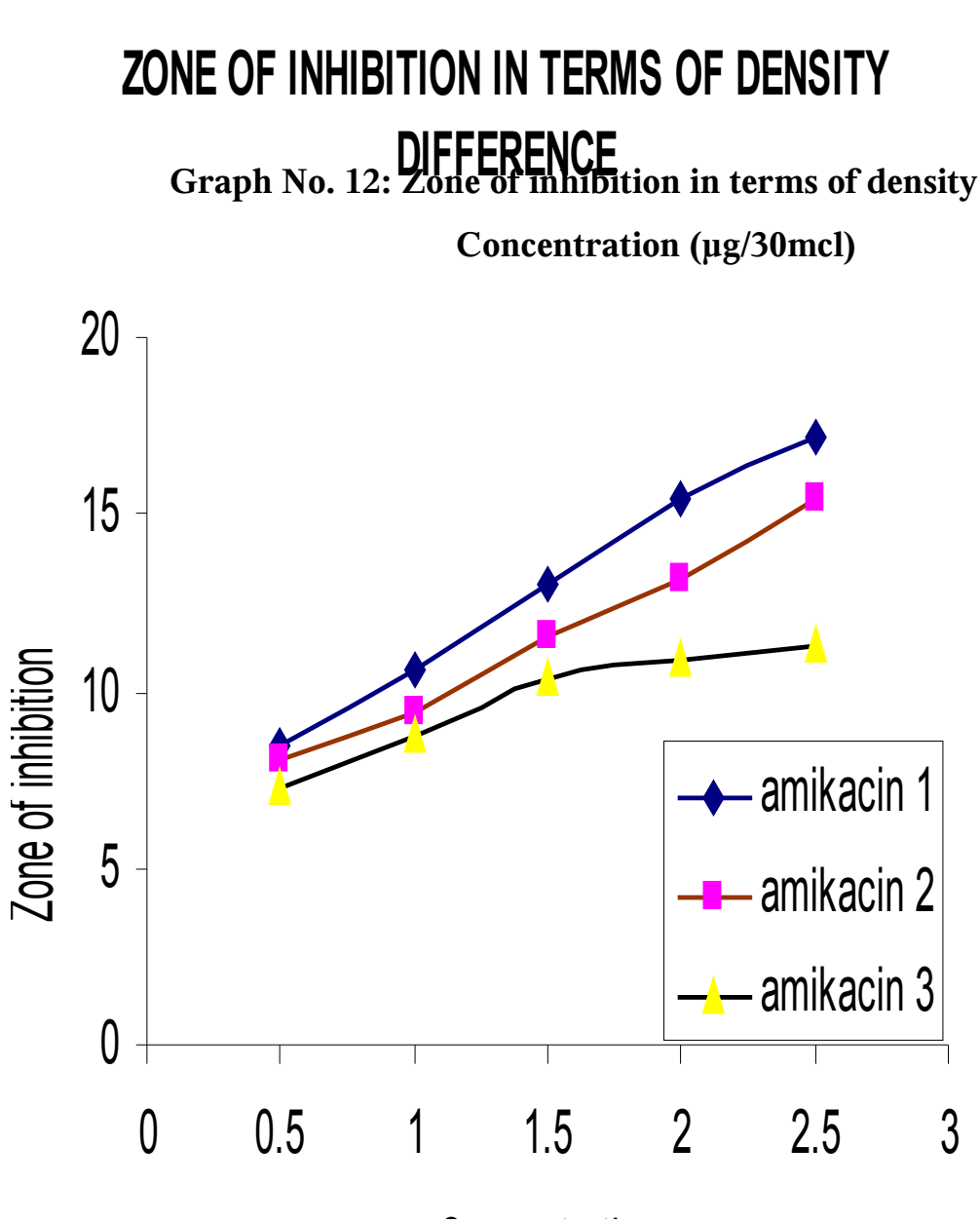
Graph No. 10: Himedia zone scale values



Concentration (µg/30mcl)

Graph No. 11: Digimizer automated values

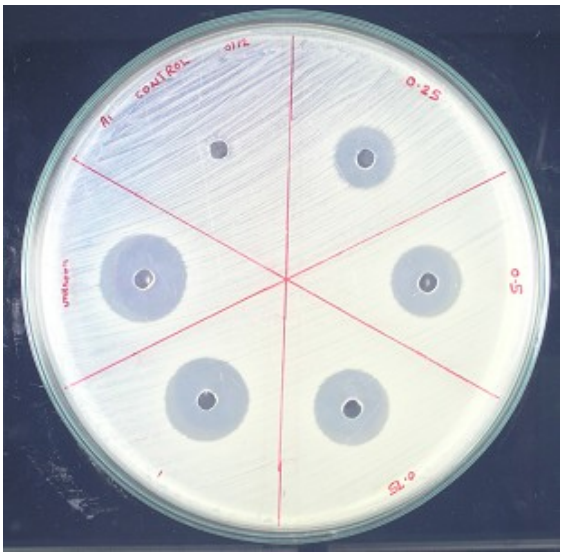




Graph No. 12: Zone of inhibition in terms of density difference

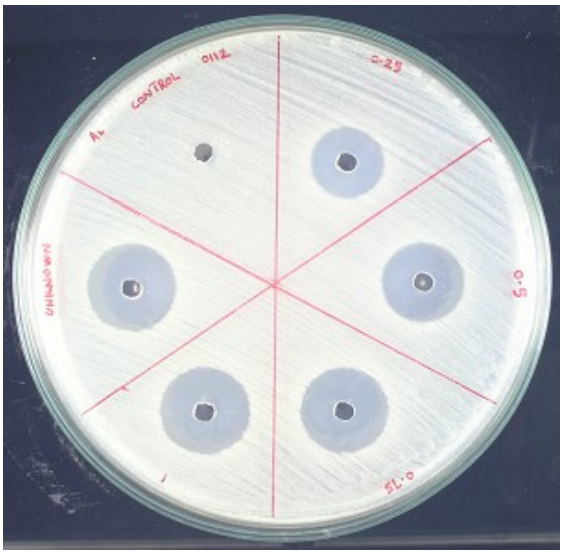
Concentration (µg/30mcl)

STAPHYLOCOCCUS AUREUS VS CIPROFLOXACIN



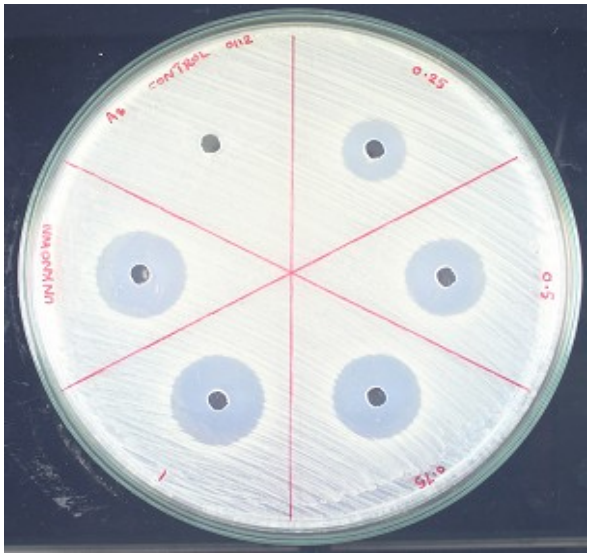
CIPROFLOXACIN 1

Fig. 5



CIPROFLOXACIN 2

Fig. 6



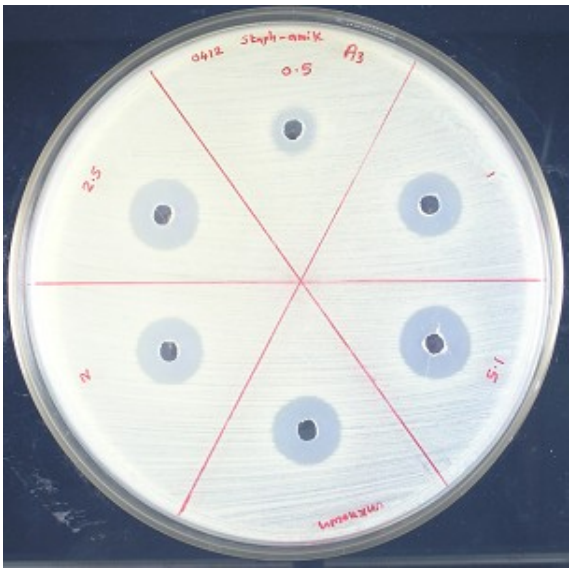
CIPROFLOXACIN 3

Fig. 7

STAPHYLOCOCCUS AUREUS VS AMIKACIN

AMIKACIN 1
Fig.8

AMIKACIN 2
Fig. 9

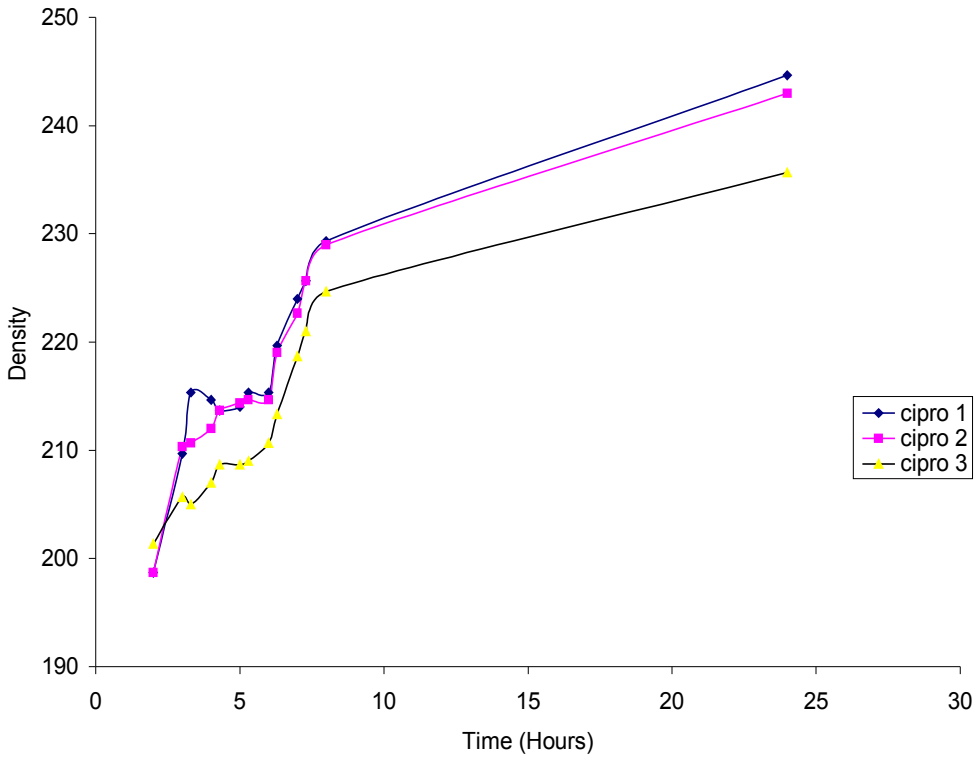


AMIKACIN 3
Fig. 10

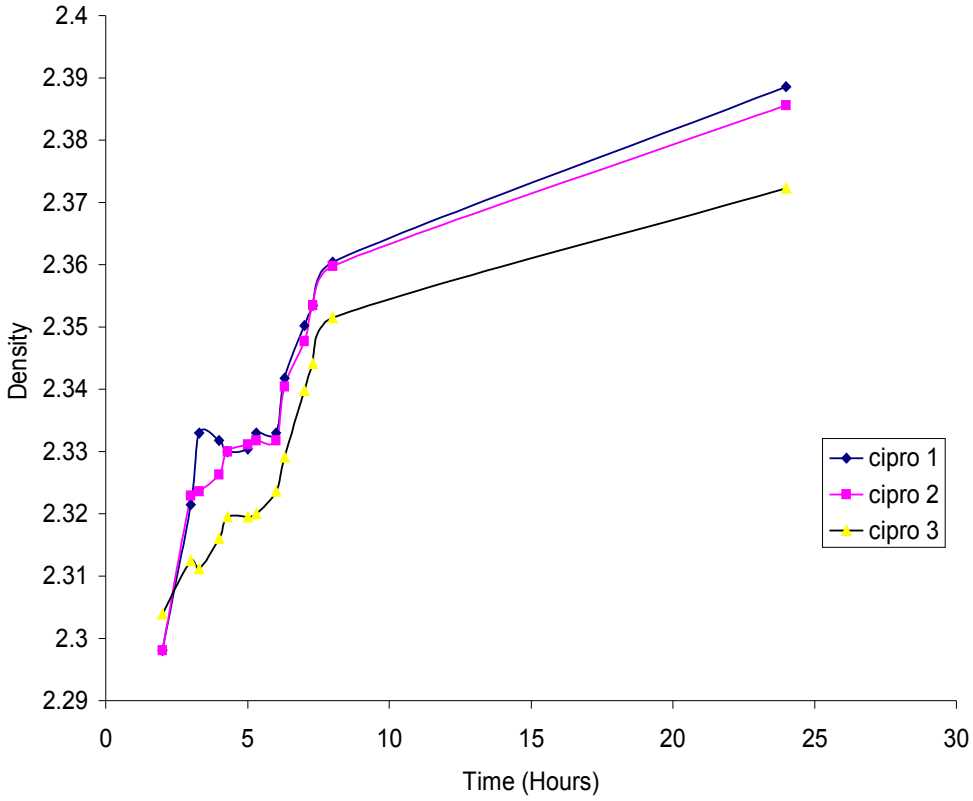
Table No. 7: Growth curve on agar plate

Sl. No.	Time (Hours)	Density of different hours					
		Cipro 1 Original value	Cipro 2 Original value	Cipro 3 Original value	Cipro1 log value	Cipro2 log value	Cipro3 log value
1	2	198.7	198.7	201.3	2.2981	2.2981	.3039
2	3	209.7	210.3	205.7	2.3215	2.3229	2.3125
3	3.30	215.3	210.7	205	2.333	2.3236	2.3111
4	4	214.7	212	207	2.3318	2.3263	2.3160
5	4.30	213.7	213.7	208.7	2.330	2.330	2.3195
6	5	214	214.3	208.7	2.3304	2.3311	2.3195
7	5.30	215.3	214.7	209	2.333	2.3318	2.320
8	6	215.3	214.7	210.7	2.333	2.3318	2.3236
9	6.30	219.7	219	213.3	2.3418	2.3404	2.3291
10	7	224	222.7	218.7	2.3502	2.3477	2.3398
11	7.30	225.7	225.7	221	2.3535	2.3535	2.3441
12	8	229.3	229	224.7	2.3604	2.3598	2.3515
13	24	244.7	243	235.7	2.3886	2.3856	2.3723

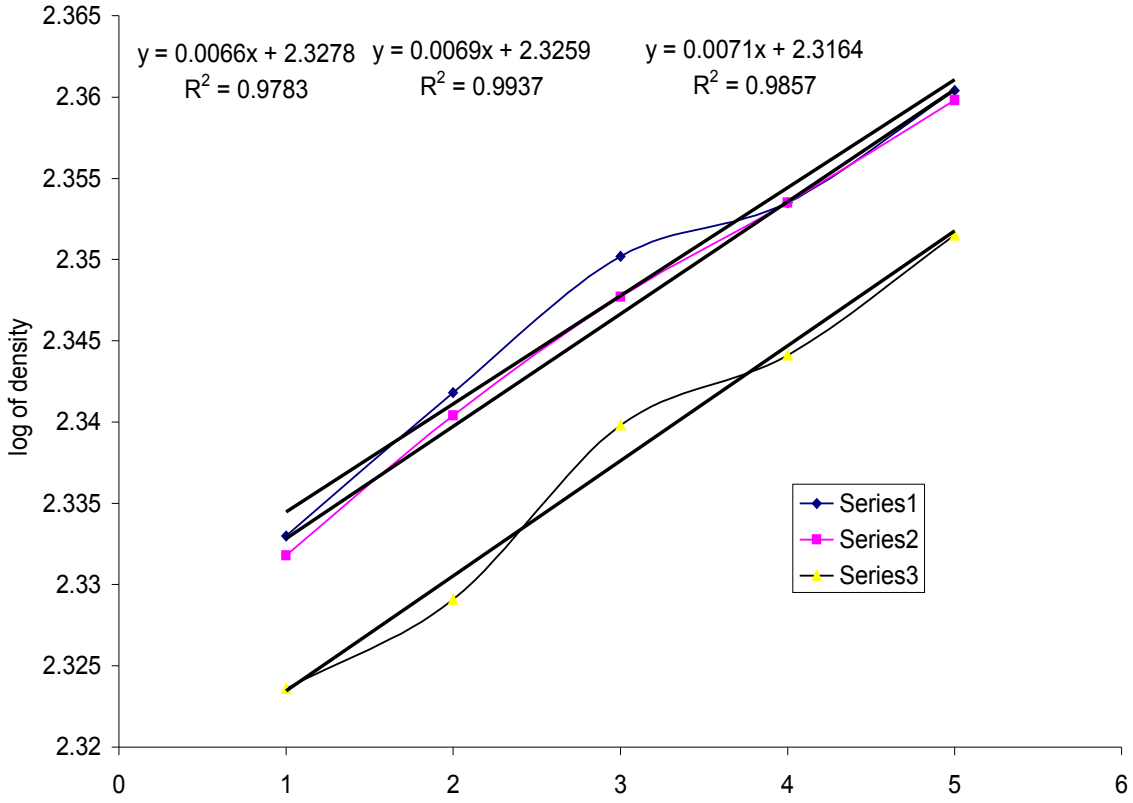
Graph No. 13: growth curve of normal values



Graph 14: growth curve of log values



Graph 15: Trend lines of linear portion



Derivation of an equation for doubling time on Petri plate

Equation for rate of growth

$$\frac{\text{Log 10 Nt-log 10N}_0}{t_t - t_0} = \mu/2.303$$

$G.T = t_t - t0/3.3(\log N_t - \log_{10} N_0)$

Ie, $G.T = \frac{1}{3.3 X m}$

$= 0.303/m$

Here,

G.T-generation time

Nt –no of organism at time t

N0-no of organism at initial time

tt-final time

t0-initial time

m-slope of the log trend line equations

slope of the 1st trend line equation is 0.0066

Therefore its generation time is 0.303/.0066 = 45.91 minutes

Slope of the 2nd trend line equation is 0.0069

Therefore its generation time is 0.303/.0069 = 43.9 minutes

Slope of the 2nd trend line equation is 0.0071

Therefore its generation time is 0.303/.0071 = 42.68 minutes

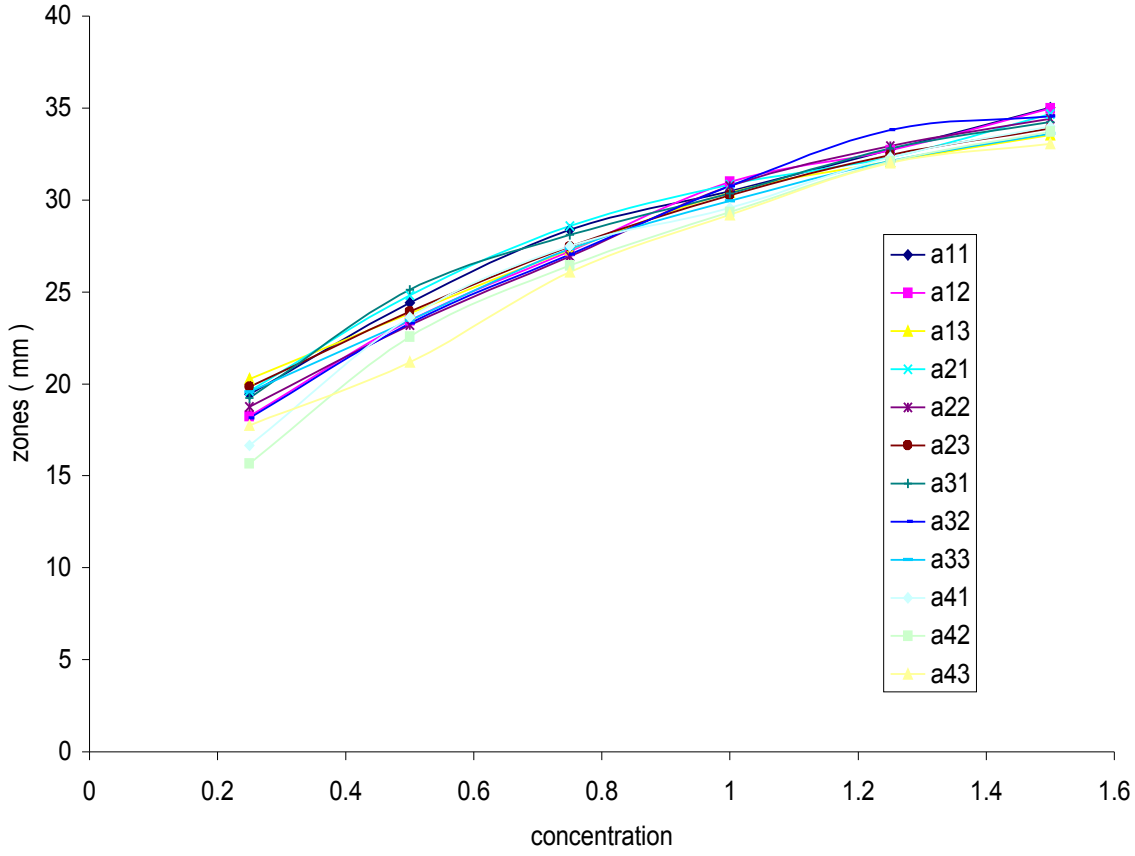
Table No: 8 CROSS CHECKING THE IDENTITY OF ORGANISM BY GENERATION TIME

Sl no	Microorganism	Generation time	Class	Disease	Symptoms	Drug of choice

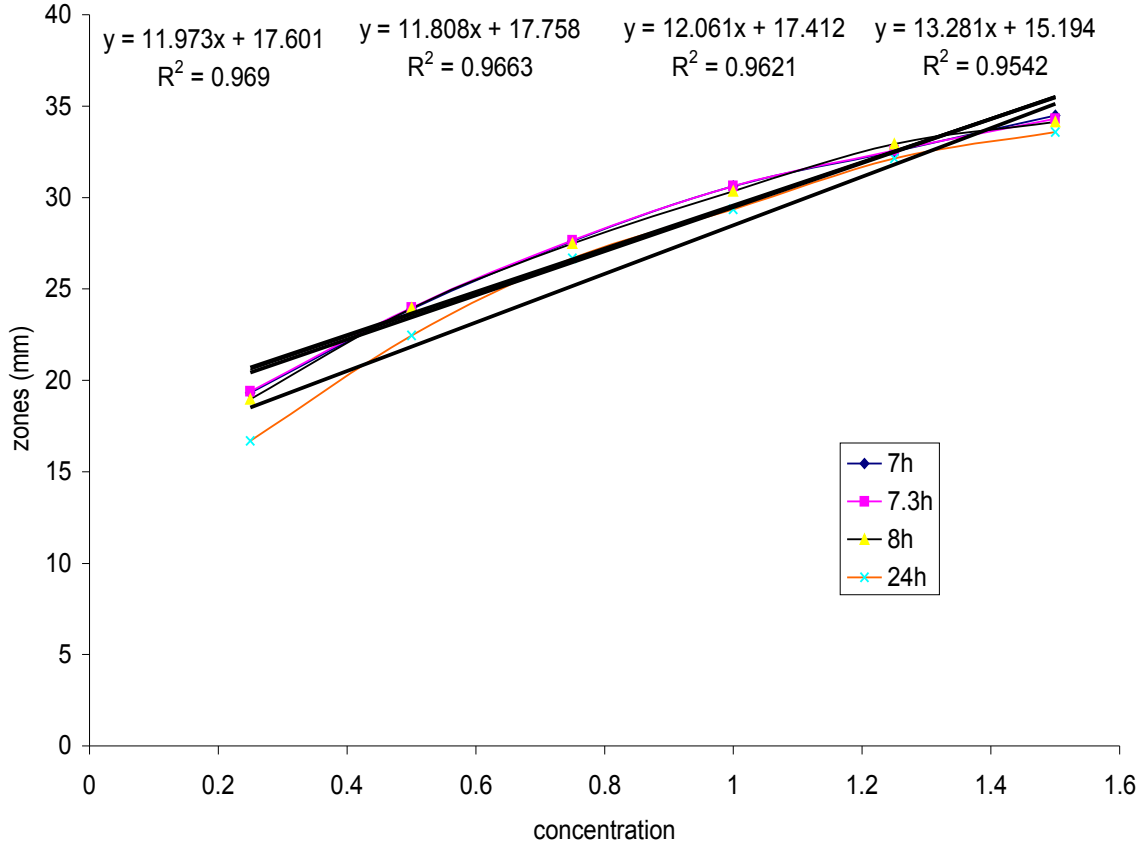
1	*E.Coli*	20	Gram –ve rod	Urinary tract infection, Local or systemic disease, Meningitis in infants	**Dysuria Lumbar pain** Hematuria Chills	Ciprofloxacin Ampicillin Cefotaxime
2	*Pseudomonas aeroginosa*	34	Gram –ve rod	Localized infection, Systemic infection	Eye.ear,skin infections Bacteremia	Tobramycin Carbenicillin Ticarcillin
3	*Bacillus species*	18-28	Gram +ve rod	Cutaneous anthrax, Pulmonary anthrax, Gastro intestinal form of anthrax	Traumatic wounds, **burns**, Inflammation on lymph nodes	Penicillin G Doxycyclin Ciprofloxacin
4	*Clostridium botulinum*	34	Gram +ve blunt ended rods	Botulism	**Paralysis,** Vomiting Diarrhoea Constipation	Penicillin G Antotoxin
5	*Staphylococcus aureus*	30	Gram +ve cocci	Skin infection, Septicemia, Osteomyelitis Endocarditis, Toxic shock syndrome,	**Abscesses**, Fever Diarrhea, hypotension	Oxacillin Nafcillin vancomycin
6	*Streptococcus species*	48	Gram +ve cocci	Acute pharyngitis, Meningitis, Acute bacterial pneumonia	Ear ache **Chest pain** Chills **Cough**	Penicillin G Clarithromycin Ceftriaxone

Table 9

Graph 16: Zone Of Inhibition 0f Cipro 1, 2, 3 At 7h, 7.30h, 8h and 24th hour



Graph 17: Average Zone Of Inhibition 0f Cipro 1, 2, 3 At 7h, 7.30h, 8h and 24th hour



Graph 18: Average Of 7h,7.30h, 8h And 24th Hour Zone Of Inhibition Of Cipro1,2, And 3

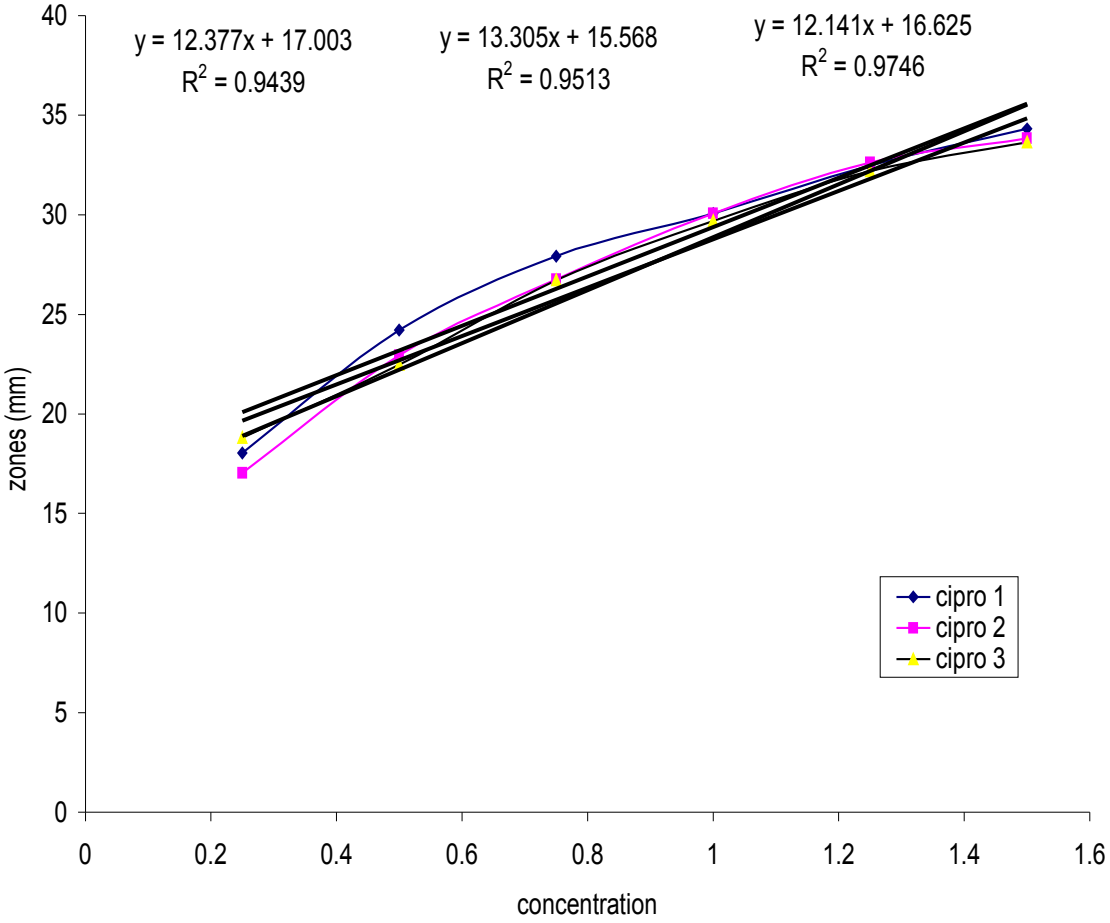


FIG :11

Time Zone Studies

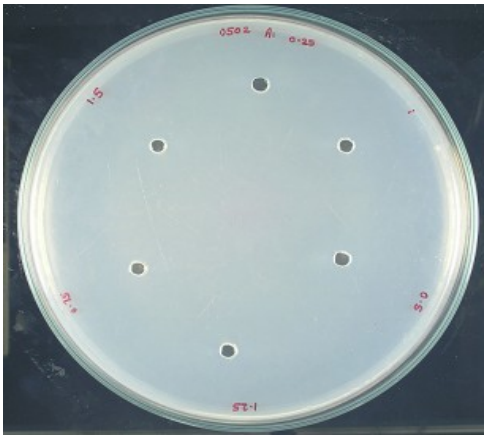


Fig :a
2 h

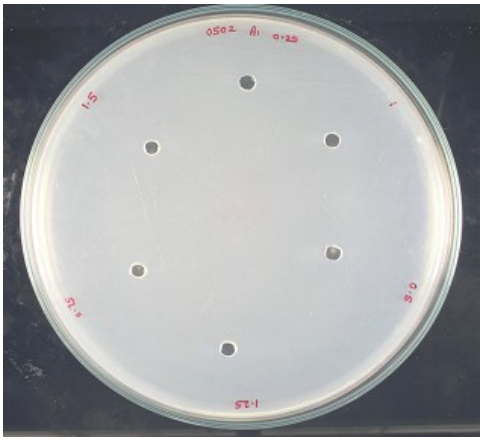


Fig :b
3 h

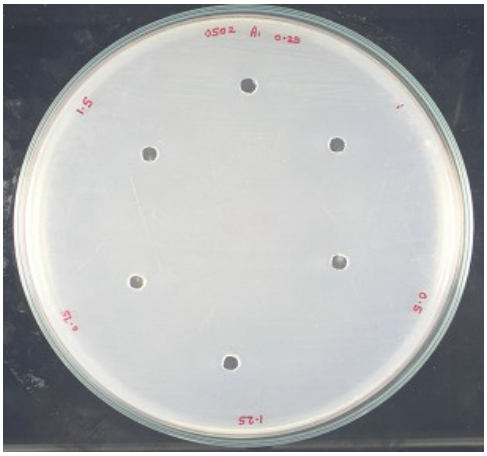


Fig :c
3.30 h

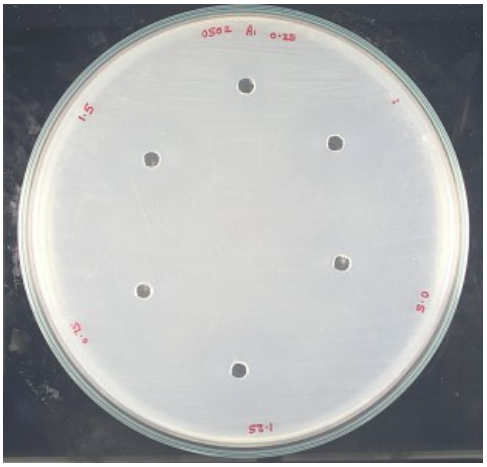


Fig :d
4 h

Time Zone Studies

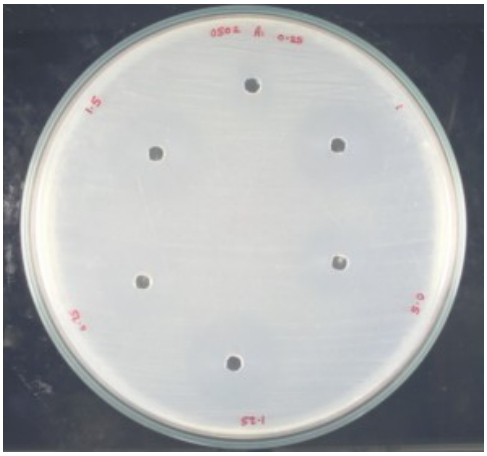


Fig :e
4.30 h

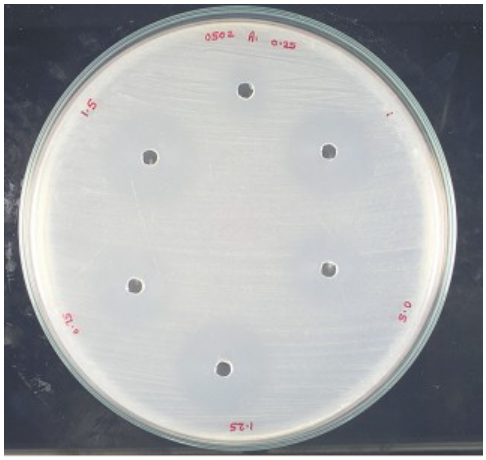


Fig :f
5 h

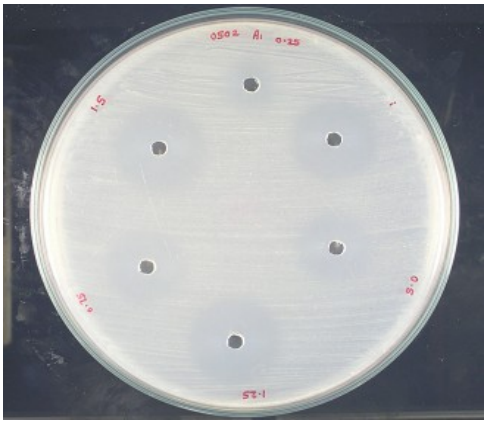


Fig :g
5.30 h

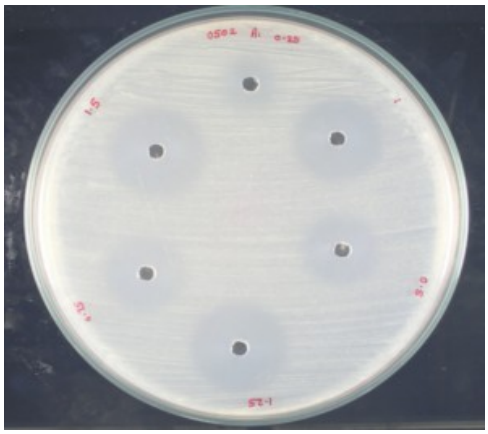


Fig :h
6 h

Time Zone Studies

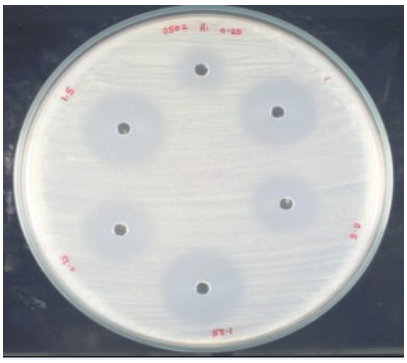


Fig :i
6.30 h

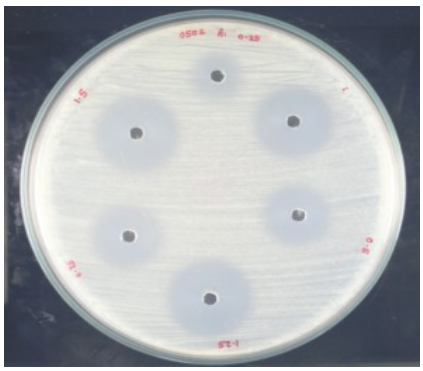


Fig :j
7 h

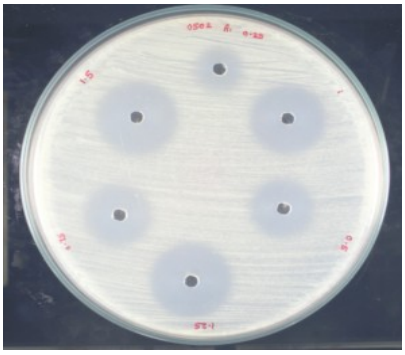


Fig :k
7.30 h

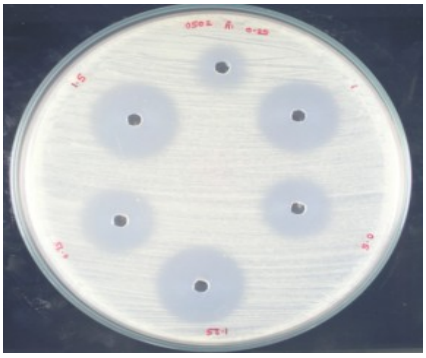


Fig :l
8 h

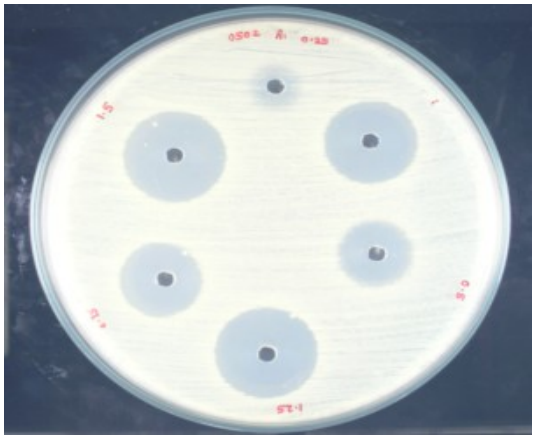


Fig :m
24 h

DISCUSSION

The macro auto calculation system, Digimizer, and digidoc methods can read, interpret and report the antimicrobial activity by agar well diffusion method.

The correlation of the zone of inhibition measurement of the himedia zone scale, automated methods of macro calculation,digimizer and digidoc systems was observed as good. The difference in the results were obtained from these four methods was negligible.

In order to compare different types of agar plate by these methods, experiment had been triplicated. The results obtained from these triplicated agar plates had been compared by graphical representation. The results of the same plate by different methods are compared by the help of MS- excel trend line equation and their r^2 values. If the r^2 values are closer to 1 it indicate the better linearity of the graph. Comparison of the different unknown predicted values was done by statics matrix.

The minimum time required for taking the zone of inhibition reading for *staphylococcus aureus* against ciprofloxacin has been studied. The zone of inhibition can be visible in between 6-8 hours. This zone of inhibition appeared is not having any significant difference on 24 hours reading.

A growth curve of *staphylococcus aureus* against ciprofloxacin had been performed. By the help of the trend line of the log density graph, derived an equation for determining the generation time on solid media

CONCLUSION

Most culture – based microbiology tasks utilize a Petri plate during processing, but rarely do the scientists or microbiologist capture the full information available from the plate. Collecting this data has been limited by the difficulties of standardizing and quantifying human observations.

An important task for the diagnostic clinical microbiology laboratory is the detection of clinically relevant antimicrobial susceptibility/resistance in individual isolates. The potential advantages of automation include standardization, which results in increased accuracy; the faster availability of results; improved data management and the possibility for the use of artificial intelligence.

Kirby Bauer disk diffusion susceptibility testing merely provides susceptibility category results (susceptible, intermediate, and resistant). On the other hand, measurement of zone sizes is tedious, time – consuming, and fraught with transcription errors.

By the help of image analysis data obtained from several centers can be utilized to monitor levels of bacterial resistance to antibiotics. Also we can eliminate the data entry errors by embedding the barcode identification systems in to the analysis software.

In this study we have developed an algorithm to automate the measurement of zone of inhibition accurately by analyzing the intensity variations within the image. On comparison with the manual method, the automated method has several advantages and hence can be considered for a widespread adaption.

We studied the rate of growth of organism on agar plate and performed the

growth curve, from the growth curve obtained, calculated the doubling time which helps for cross checking the class of organism during susceptibility testing. Also attempted to determine the antibiotic susceptibility of microorganism with reduced duration of time to facilitate an early initiation of treatment.

A computer based automated image analysis method can be used to perform complicated image processing functions to bring out relevant features like morphological characters that may provide very useful informations but are difficult to extract by conventional means.

ABBREVIATIONS

M.I.C	MINIMUM INHIBITORY CONCENTRATIONS
H.M.Z.S	HIMEDIA ZONE SCALE
D.M	DIGIMIZER
D.D	DIGIDOC
1-D	1-DIMENSIONAL
I.D.V	INTEGRATED DENSITY VALUES
AVG DENSITY	AVERAGE DENSITY
P.A.E	POST ANTIBIOTIC EFFECT
S.R.D	SINGLE RADIAL IMMUNO DIFFUSION
B.M.D	BROTH MICRO DILUTION
CMEIAS	CENTRE FOR MICROBIAL ECOLOGY IMAGE ANALYSING SYSTEM
E.P.R	END POINT RATIO
AWDA	AGAR WELL DIFFUSION ASSAY
E.TEST	EPSILOMER TEST
NCIM	NATIONAL COLLECTIONS FOR INDUSTRIAL MICRO ORGANISM
I.A.S	IMAGE ANALYSING SYSTEM